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**Differential role of the endothelium in regulating microvascular
blood flow**

Tang, Tao, Ph.D.

East Tennessee State University, 1994

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**300 N. Zeeb Rd.
Ann Arbor, MI 48106**

**DIFFERENTIAL ROLE OF THE ENDOTHELIUM IN REGULATING
MICROVASCULAR BLOOD FLOW**

A Dissertation
Presented to
the Faculty of the Department of Physiology
James H. Quillen College of Medicine
East Tennessee State University

In partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

By

Tao Tang

May 1994

APPROVAL

This is to certify that the Graduate Committee of


Tao Tang

met on the

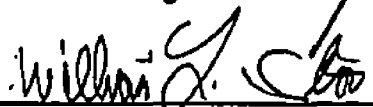
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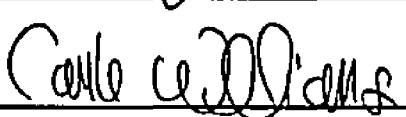
The committee read and examined her dissertation, supervised her defense of it in an oral examination, and decided to recommend that her study be submitted to the Graduate Council and the Associate Vice-President for Research and Dean of the Graduate School, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Science.


Chairman, Graduate Committee









Signed on behalf of
the Graduate Council



Associate Vice-President for
Research and Dean of the
Graduate School

ABSTRACT
DIFFERENTIAL ROLE OF THE ENDOTHELIUM IN REGULATING
MICROVASCULAR BLOOD FLOW

by

Tao Tang

The vascular endothelial cell (EC) plays an important role in regulating vascular tone and local blood flow by sensing chemical and mechanical stimuli on the vascular wall and releasing a host of vasoactive substances upon activations of endogenous or exogenous vasoactive substances. The central hypothesis is that local control of blood flow and autoregulatory behavior in the microcirculation is distinctive at different levels of the vasculature and is dependent on the cellular activities of the EC and its interaction with the local environment. The *in vivo* as well as the *ex vivo*, flow-controlled preparations of the hamster cheek pouch were utilized. Inhibition of Endothelium-Derive Relaxing Factor (EDRF) synthesis and the functional impairment by light-dye (L-D) treatment were used to remove functional characteristics of the EC. It is found that the EC played differential roles in modulating vascular tone and blood flow in distinct segments of arterioles. Impairment of the EC by L-D treatment significantly reduced both acetylcholine (Ach)-induced dilation and the local angiotensin conversion in small (4th order) arterioles (A_4). Whereas, data obtained after inhibition of EDRF synthesis indicated that EDRF pathway appeared to be the dominant regulatory mechanism mediating agonists (e.g. Ach)-induced responses in these small vessels. In large (2nd order) arterioles (A_2), on the other hand, neither L-D treatment nor EDRF inhibition affected Ach-induced dilation or local angiotensin conversion; therefore, these responses seemed to be independent of the EC or EDRF pathway. Autoregulation was observed in both A_2 and A_4 when perfusion flow (shear stress) and perfusion pressure (stretch) were elevated. Nevertheless, the underlying regulatory mechanisms in response to mechanical stimuli differed in these series-arranged arterioles. The EC/EDRF-dependent, flow-induced dilation was dominant in A_2 ; whereas, the myogenic autoregulation (which appears to be independent of the EC) played major role in A_4 . Therefore, the function of the EC does not appear homogenous throughout the arteriolar portion of the microcirculation. Thus, the local control of blood flow and autoregulatory behavior in the microcirculation is distinctive at different levels of the vasculature; whereas, the differential role of the EC in discrete segments of series-arranged arterioles seems to be the determinant for these differences. These differential modulations of vascular tone and blood flow by the EC at discrete levels of the microcirculation may have important implications in pathological conditions, such as hypertension, diabetes, and atherosclerosis.

DEDICATION

This dissertation is dedicated to my dear husband Xiang Ren for his faithful love, continuous support and immeasurable sacrifice that have allowed me to study far away from home during these four solid years; and to our parents and our daughter Mengyao for their love and understanding. Without their support this dissertation would not have been possible.

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CHAPTER 1

INTRODUCTION

The microvasculature is the terminal portion of the vascular tree beginning with the arterioles. The term "microcirculation" is used to designate blood flow through microvessels including the arteriole, the terminal arteriole, the capillary, the post capillary venule and the venule. The purpose of the microcirculation is to deliver blood to the parenchymal constituents of the tissue in accord with their metabolic activity. This is accomplished by allowing a given volume of blood to perfuse the terminal network of vessels at a rate that is compatible with an adequate exchange of fluids and materials across the blood-tissue interface. The key functions of the microcirculation include: (1) the capacity to adjust blood flow with metabolic requirements of the tissue, and (2) the local autoregulatory adjustments that serve to stabilize flow as well as pressure. Maintaining these functions requires special intrinsic mechanisms since the obvious physical features such as the lesser distensibility of the thick-walled arterioles compared to small arterioles, the non-newtonian blood flow through the narrow capillaries, or the repeated dichotomies and interdigitation of the microvasculature cannot explain the striking differences between the behavior of large and small blood vessels. Therefore, the microcirculation, indeed, is considered as an independent organic unit with intrinsic mechanisms for the local regulation of blood flow (Zweifach, 1977).

The general principle of hemodynamics is the basis of any control and it is described by the law of flow: $Q = \Delta P / R$ where the flow, Q , depends on the driving force ΔP , which is the "pressure drop" down the circulatory bed, and R is the resistance to flow for a particular vascular bed. This resistance is determined by the geometry of the

vessels involved in that particular route for blood flow, i.e., their length (l), diameter ($d=2r$), as well as the viscosity (η) of the fluid flowing through these vessels. According to Poiseuille's Law, the resistance is given by: $R=\Delta P/Q=(8/\pi)(\eta)(l/r^4)$. Since the viscosity will be the same (or approximately so) for different vessels, whereas R is inversely related to the 4th power of the radius of the vessel, the diameter of the vessel is the major factor contributing to the relative resistance of the different vessels. Therefore, control of the distribution of blood flow can be determined by two factors: 1) by controlling the driving pressure (ΔP), and 2) by controlling the diameter of the vessel, thus the resistance to flow (R), of each vascular bed (Burton, 1972).

The mechanisms involved in regulating microvascular blood flow via changes in resistance, e.g. diameter of microvessels, can be divided into four general categories: neural, humoral, metabolic, and myogenic controls. The relative importance of each of these mechanisms in controlling blood flow to a tissue is determined somewhat by the function of the tissue. In addition, studies within the last 10-15 years have revealed a crucial role for the endothelial cell in the local control of blood flow, not only in large arteries but also at the level of microvessels. Therefore, a new regulatory category can be considered as the endothelial-dependent vasoactive response. This study concentrates on the role of endothelial cells in regulating microvascular blood flow and their interaction with other local regulatory mechanism(s), such as myogenic control, in distinct segments of series-arranged arterioles in the hamster cheek pouch.

Local Regulation of Blood Flow, the Myogenic Response

The myogenic theory was proposed by Bayliss (1902) who was the first to suggest that distention of blood vessels is actually a stimulus for the contraction of

smooth muscle cells in the vessel wall. Since then, numerous microcirculatory studies have been made in an attempt to demonstrate the myogenic behavior of blood vessels in the microcirculation. Klabunde and Johnson (1977) demonstrated that a very brief period of arterial occlusion (as short as 3 seconds) led to substantial vasodilation. It seemed unlikely that reduction in blood flow for such a short period could produce significant metabolic changes. Therefore, the observed vasodilation could have resulted from a reduction in intra-arterial pressure. Secondly, a reduction of ambient pressure around an organ produced sustained vasoconstriction (Greenfield, 1964). Presumably this occurred due to an increased pressure gradient across the blood vessel wall, since the arteriole-venous pressure difference did not change. Furthermore, elevation of venous pressure caused a sustained increase in precapillary resistance in some organs, e.g. intestine, liver, and skeletal muscle. This vasoconstriction was not due to neural reflex activity or tissue pressure changes (Johnson, 1958), and was not a metabolic effect, since blood flow decreased (Mellander, *et al.*, 1964). Lastly, elevation of static intravascular pressure in the arterioles during no-flow conditions resulted in a sustained contraction of arterioles in the mesentery (Johnson and Intaglietta, 1976). These studies provided a variety of evidence at the level of microvasculature supporting the myogenic hypothesis.

In summary, Johnson (1981) defined the myogenic response as the behavior that the vascular smooth muscle cell "senses" physical changes (stretch or tension) and responds by contracting/or relaxing. According to the myogenic theory, the smooth muscle cells in the vessel wall partially contract in response to distention, even under conditions when there is no innervation or exposure to blood borne vasoactive agents. Since the vasculature normally is in a state of partial constriction, vascular resistance

would be decreased as the vessels relax when distention decreases, or be increased in response to an increase in transmural pressure. In both cases the myogenic responsiveness of the blood vessels would tend to keep blood flow constant. Hence, the benefit of this reaction is to maintain a constant blood flow to the tissues irrespective of the level of arterial pressure.

Mechanisms of the myogenic response

The nature of the vascular myogenic response to pressure is still unclear. Two mechanisms, however, have been proposed to describe the myogenic response. First, Folkow (1964) has suggested that passive stretch leads to an increase in the spontaneous firing rate of the smooth muscle cell. In this model, smooth muscle cells were considered under a spontaneous "pacemaker" myogenic activity, i.e. spontaneously contracting and relaxing at a certain frequency. Elevation of intravascular pressure lengthens the smooth muscle cell and hastens the onset of the next contraction. When the cell contracts and shortens, it is no longer stretched beyond its normal length, and the stimulus for contraction presumably disappears; thus, the muscle cell relaxes. However, as the muscle cell relaxes, the elevated pressure causes the cell to be stretched beyond its normal resting length, which brings on a second contraction earlier than it would occur normally. As a consequence the smooth muscle cell fires more frequently and it spends more time in the contracted state. Thus, the time-averaged vessel diameter was actually decreased. According to Folkow's theory, the vascular smooth muscle cell functions as a "length receptor" to sense the mechanical stimulus.

The other hypothesis suggests that the smooth muscle cell is a tension receptor, rather than a length receptor. Bülbbring (1955) reported that elongation of a strip of

guinea pig taenia coli caused membrane depolarization and increased spike activity. The electrical changes correlated closely to tension but poorly to length. This observation is especially important in considering vascular smooth muscle, since circumferential tension (T) in the vessel varies as a function of both intravascular pressure (P) and vessel radius (r), as described by La Place Law ($T = P \times r$). According to the control mechanism based on servo-control of circumferential tension, an increase in pressure leads to an elevation of wall tension, which elicits a myogenic contraction. As the contraction proceeds, radius decreases, and tension also falls. Thus, the proposed mechanism incorporates a negative feedback system that limits the magnitude of the contraction elicited by pressure elevation. If the control system were geared to maintain a perfectly constant wall tension, vessel radius would decrease in proportion to the pressure increase. In this case, the control system would have a closed-loop gain of unity. If the gain were less than unity, obviously the response would be less also. Therefore, the vessel radius might increase, decrease, or not change when intravascular pressure is elevated depending on the gain of this system. This hypothesis was supported by the study of Bouskela and Wiederhielm (1979) in which they found that increased transmural pressure in the arteriolar network led to a reduction in vessel radius sufficient to maintain wall tension very nearly constant. Studies of mesenteric arterioles during autoregulation also indicated that wall tension changes were consistent with this hypothesis (Johnson and Intaglietta, 1976). Furthermore, some studies did find the gain differences along the vascular tree, in which the distal branches of the arterioles have a higher myogenic response gain compared to the proximal branches (Jackson and Duling, 1989; Davis, 1991). Accordingly, the regulation of the vascular resistance by myogenic

mechanism may not be homogeneous throughout the vascular tree, since there may be differences in the spontaneous firing rate, the basal vascular tone, and the gain of the myogenic response in distinct segments of series-arranged arterioles.

Cellular mechanisms involved in the vascular myogenic response

Although information about the cellular mechanisms underlying the myogenic response has been slow to accumulate, there have been several broad hypotheses concerning the sequence of events that couple changes in intravascular pressure or stretch with alterations in vascular smooth muscle activation. These hypotheses include 1) altered membrane properties leading to activation of ion channels; 2) modulation of biochemical cell signaling pathways within vascular smooth muscle; and 3) length-dependent changes in contractile protein function.

Activation of ion channels. There are several lines of evidence supporting the first hypothesis. Kirber *et al.* (1988) have characterized a nonselective cation stretch-activated (SA) channel in frog visceral smooth muscle. They found that in membrane patches that contained both SA channels and Ca^{2+} -activated K^+ channels, pipette suction, i.e. the pressure application, appeared to gate specifically the SA channel. Meanwhile, their data indicated that the SA channel had a low open probability at rest, which increased over 10-fold when transpatch pressure was increased from 0 to 10 mmHg. Likewise, Davis *et al.* (1992) have showed that in smooth muscle cells from pig coronary artery, patch pipette suction (-5 to -40 cmH₂O) increased open probability of a nonselective cation channel but had no effect on open probability of a Ca^{2+} activated K^+ channel. A similar channel has been identified also in smooth muscle cells from rabbit

pulmonary artery (Kirber *et al.*, 1989). Since the membrane impedances were very high at rest, $>1\text{-}2\text{ G}\Omega$ in visceral smooth muscle cells (Kirber *et al.*, 1988) and vascular smooth muscle cells (Davis *et al.*, 1992), gating of these SA channels should significantly depolarize the membrane. This depolarization could in turn activate voltage gated calcium channel (VGCC), which have also been identified in these cell types (Vivaudou *et al.*, 1988; Matsuda *et al.*, 1990). In addition, Ca^{2+} could directly enter the cell through SA channels, thus directly activate the contractile machinery. Hence, the opening of the SA channels may be the initial event of the transduction of stretch by vascular smooth muscle cells (Meininger and Davis, 1992).

Modulation of biochemical cell signalling pathways. Several biochemical signalling pathways may be implicated in the myogenic responses including elevation of intracellular calcium, initiation of inositol phosphate cascade, activation of protein kinase C (PKC) and myosin light chain kinase (MLCK), and the modulation of cAMP level in the cells. It is well known that removal of extracellular calcium eliminates myogenic responsiveness (Jackson and Duling, 1989) and the requirement of extracellular Ca^{2+} entry for myogenic responsiveness has been clearly demonstrated (Laher *et al.*, 1988). In these studies, myogenic tone was associated with the uptake of $^{45}\text{Ca}^{2+}$ and that this tone rapidly decreased in Ca^{2+} -free bathing solutions. A recent study demonstrated further that there was a sustained increase in intracellular Ca^{2+} with stretch, and the relation between the peak $[\text{Ca}^{2+}]_i$ change and the magnitude of stretch was sigmoidal (Davis *et al.*, 1992b). In addition, this relation was shifted upward in 10 mM Ca^{2+} bathing solution and abolished with prolonged exposure to Ca^{2+} -free solution. These results provided direct evidence supporting the obligatory role of Ca^{2+} in myogenic responses.

The mechanical activation of vascular smooth muscle cells also may involve phosphoinositol hydrolysis as occurs during receptor-mediated cell activation, in which the breakdown of phospholipids by phospholipase C leads to the formation of inositol 1,4,5 trisphosphate (IP₃) and diacylglycerol (DAG). Consequently, IP₃ could cause Ca²⁺ release from intracellular stores; while, DAG in turn could activate PKC (Bansal and Majerus, 1990). Studies supporting this hypothesis illustrated that there was a transient increase in IP₃ level in cultured pulmonary vascular smooth muscle cells when these cells underwent a 20% stretch (Kulik *et al.*, 1988). Harder *et al.* (1991) studied pressurized arterial segments isolated from renal circulation and demonstrated that IP₃ increased markedly following a step change in intravascular pressure. Meanwhile, a concomitant threefold increase in DAG was also observed. These findings suggest that inositol phosphate cascade can be stimulated by mechanical events also known to initiate the myogenic responses, and thus, play a role in the signal transduction in the myogenic response.

As mentioned earlier, the activation of PKC is one of the consequences of phosphoinositol hydrolysis. Therefore, PKC activation is actually a part of the inositol phosphate cascade. In this regard, PKC activation might be expected to play a role in the myogenic response since IP₃ and DAG elevation during stretch stimuli were observed as mentioned above. This postulate has been studied by Laher and Bevan using activators and inhibitors of PKC. They found that activation of PKC with phorbol ester selectively enhanced stretch-induced tone; whereas, inhibition of PKC with staurosporine inhibited the stretch-induced tone (Laher and Bevan, 1987; Laher and Bevan, 1989). Other studies illustrated that indolactam, a PKC activator, augmented basal vascular tone but was

without effect on the constrictor response elicited by a rapid change in pressure (Osol *et al.*, 1991). It is difficult to specifically assess the role of PKC on myogenic response to acute changes in pressure if basal vascular tone, i.e., smooth muscle cell length and/or wall stress were altered. Nevertheless, the directionality of the changes seen with those pharmacological agonists and antagonists make a role for PKC seem credible, although the intracellular targets of PKC that are of relevance to the myogenic response have yet to be identified.

It is well known that myosin light-chain kinase (MLCK) plays an important role in contractile activation of smooth muscle by phosphorylating myosin light chains. As demonstrated in studies of Ledvora *et al.* (1983), stretch of the strips of porcine carotid artery resulted in an increase in phosphorylation of myosin light chains. This stretch-induced phosphorylation of myosin light chain was insensitive to the voltage-operated calcium channel agonist (verapamil), to bathing the preparation in Ca^{2+} -free solution or to short-term exposure to ethyleneglycol-bis(β -aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). Long-term washing with EGTA inhibited the stretch-induced phosphorylation. These data suggested that stretch-induced calcium release was responsible for activation of MLCK. More recently, Nakayama and Tanaka (1988) measured increase in tension development and Ca^{2+} level following stretch in helical strips of the canine cerebral artery. They found that after treatment with a calmodulin inhibitor or a MLCK inhibitor, increase in tension was reduced to a greater extent than the Ca^{2+} signal. These results are in accordance with the postulate that Ca^{2+} , calmodulin and myosin light chain phosphorylation are involved in the signaling sequence for the stretch-induced responses.

Increase in cAMP has been known to cause relaxation in vascular smooth muscle cells. This fact predicts a contraction if cAMP level were reduced. This is indeed what Mills and colleagues reported (Mills *et al.*, 1990). They found that cyclic stretch of cultured vascular smooth muscle cells from coronary artery reduced the activity of adenylate cyclase. Although there are not many data available in this regard, these results are representative of what occurs in the intact blood vessel wall during changes in intravascular pressure, i.e., a decrease in cAMP promotes constriction during elevations of intravascular pressure.

The mechanisms responsible for linking these second messengers to the physical stimulus have not been identified, but elements making up the mechanical linkages between the extracellular matrix and the cells' cytoskeletal or contractile machinery are good candidates. Membrane proteins with multiple membrane spanning domains are likely sites for mechanotransduction (Meininger and Davis, 1992). To what extent these biochemical cell signalling mechanisms operate independently of the ionic events at the membrane remains to be determined.

Contractile protein functions. The linkage between second messengers and the physical stimulus may be bridged by contractile protein of smooth muscle cells. Using permeabilized molluscan smooth muscle, Ridgway *et al.* (1983) demonstrated that the Ca^{2+} sensitivity of the myofilaments changed as a function of cell length, i.e. the level of contraction modulated the calcium sensitivity of the muscle. The mechanism of this effect appears to be a cross-bridge-induced change in the calcium affinity of the myofilaments. This is due to the fact that there is period of time, termed "latch state", in which force generation can be maintained despite a reduction in myosin light chain

phosphorylation and it reflects a population of slow-cycling, non-phosphorylated cross bridges (Murphy, 1980). Hence, alterations in contractile filament overlap or latch state may directly result in changes in smooth muscle contractility or calcium sensitivity. Although it is not clear whether PKC or MLCK were involved in this event, it is conceivable to postulate a role for these contractile proteins in the stretch-induced myogenic responses.

Sequence of the intracellular events. There are still many gaps in our knowledge regarding the cellular mechanisms responsible for the myogenic response; however, there is sufficient evidence to postulate a plausible sequence of intracellular events. According to the scheme proposed by Meininger and Davis (1992), vascular smooth muscle stretch acts primarily at the cell membrane to alter ion conductance and perhaps initiate hydrolysis of membrane phospholipids. The change in membrane ion conductance begins with increased flux of Na^+ , K^+ , and Ca^{2+} through SA channels. This influx of cations results in membrane depolarization, which would act to recruit voltage-operated calcium channels augmenting calcium influx. Ca^{2+} influx through SA channels could directly initiate contraction or act to trigger Ca^{2+} release from internal stores. After the calcium response, myosin light chain activation would initiate contraction. In parallel with these ionic events, a stretch-induced breakdown of membrane phospholipids may provide sufficient IP_3 to stimulate Ca^{2+} release from intracellular stores, thus contributing to the initial Ca^{2+} rise. Concomitant activation of PKC through DAG is hypothesized to phosphorylate contractile proteins leading perhaps to an enhanced Ca^{2+} sensitivity. The involvement of these intracellular pathways may vary from tissue to tissue or from species to species; however, principles described above would aid in our understanding

of the interactions between myogenic and other regulatory mechanisms.

Functional concepts of myogenic control

Myogenic responses have been seen in many tissues including cat mesentery, rat cremaster and hindquarter, porcine coronary vessels, cat cerebral arteries, hamster cheek pouch, and bat wings etc. (Johnson, 1981). The extensiveness of this response establishes a fundamental regulatory role for this mechanism in the circulatory system. Analyzing the results of numbers of *in vivo* studies, Mellander (1989) summarized that myogenic regulatory mechanisms, directly or indirectly, contribute to circulatory homeostasis by exerting the following main functions :

- (1) A tonic excitatory function on the vasculature evoked by continuous static and dynamic blood pressure stimuli, which serve as triggers for the development and maintenance of a pronounced intrinsic myogenic basal vascular tone, especially in arterial microvessels. It is on this intrinsic tone that the other vascular control systems operate to exert their functions.
- (2) Establishment of the pronounced intrinsic myogenic tone, which serves to maintain normal arterial pressure in the systemic circulation in the resting organism.
- (3) Improvement of nutritional flow and exchange exemplified by: (a) blood flow recruitment in individual organs in relation to their functional states, made possible by graded inhibition of intrinsic myogenic tone in the resistance vessels; (b) capillary recruitment made possible by inhibition of myogenic tone in terminal arterioles/precapillary sphincters, thereby adjusting the exchange function to the local tissue demands; (c) adjustments to the capillary perfusion: diffusion ratio towards an optimal exchange function in the structurally heterogeneous network of capillaries with

different bore and length, accomplished by myogenic regulation of precapillary

'sphincter' tone; (d) postocclusive reactive hyperaemia accomplished by

myogenic/metabolic inhibition of intrinsic tone in the resistance vessels.

(4) Protection against the harmful circulatory effects of changes in blood pressure, exemplified by: (a) autoregulation of blood flow during changed arterial pressure, accomplished by resistance vessel adjustments; (b) autoregulation of capillary hydrostatic pressure during changed arterial pressure, accomplished by adjustments to the pre- to postcapillary resistance ratio; (c) autoregulation of transcapillary filtration during hydrostatic load on the vascular bed, accomplished by constriction of terminal arterioles/ precapillary sphincters, decreasing the functional capillary surface area available for filtration.

It should be pointed out that the myogenic mechanism is not the only factor that contributes to the development of normal vascular tone. At any one time, the smooth muscle cell may be excited by neural, humoral, phasic mechanical, and sustained mechanical stimuli. Conversely, beta-adrenergic, ionic and metabolic factors oppose the excitatory stimuli. The smooth muscle cell senses and responds to these multiple inputs by integrating the overall signals; therefore, the control system can at times be coordinated, giving rise to periodic contraction and relaxation.

Is myogenic response endothelium-dependent?

Since Bayliss first proposed the myogenic response in 1902, many evidences have been shown that this response is not mediated by nerves, vasoactive metabolites, or circulating vasoactive substances (Folkow, 1949; Hwa and Bevan, 1986; Kulik *et al.*, 1988). Recently, the myogenic concept, that is, pressure-induced contraction originating

in vascular smooth muscle, has been challenged by studies that show the response to be dependent on an intact, functional endothelium. Harder (1987) determined in vitro pressure-diameter relations for cat middle cerebral arteries in physiological saline solution (PSS) and in calcium-free saline solution (CFS). He demonstrated that cerebral arteries showed modest myogenic responses. In PSS, vascular diameters were relatively unchanged over a pressure range of 40-160 mmHg compared with passive tensions in CFS over the same pressure range. However, the destruction of endothelial cells via enzyme (collagenase and elastase) perfusion abolished responses seen under the control condition implying a dependence of an intact endothelium in mediating the activation of isolated cat cerebral arteries in response to a changing transmural pressure. Katusic *et al.* (1987) reported that stretch applied to isolated canine basilar arteries caused the development of active tension in rings with endothelium but not in those endothelium-removed preparations. Furthermore, they demonstrated that this endothelium-dependent response to stretch was abolished with indomethacin suggesting that endothelium may contribute to the autoregulation of cerebral blood flow during increases in transmural pressure by the release of prostaglandins. Rubanyi (1988) devised a bioassay system in which a ring of denuded coronary artery was superfused by the effluent from a perfused carotid artery segment. Pressurizing endothelial intact upstream carotid segment from 0 to 35 mmHg initiated active force development of both tissues. Removal of the endothelium from the carotid segment or treatment of that segment with EDRF inhibitor, methylene blue, prior to the pressurization eliminated subsequent contraction of the downstream bioassay tissue. These results suggested that the pressure induced contraction of canine carotid arteries was mediated by depression of EDRF synthesis/or

release. Another possible mechanism for endothelial cells to mediate stretch-induced response in cerebral arteries is through release of a contractile substance when stimulated; this concept was supported by the study of Harder *et al.* (1989).

Most studies, however, present results favoring the hypothesis that the myogenic response is an intrinsic characteristic of the smooth muscle which originates in smooth muscle cells. This is especially true for arterioles, the most myogenically responsive vascular segments and the most important segment in terms of vascular resistance, since no studies to date have demonstrated that endothelial cells are required for myogenic responsiveness (Meininger and Davis, 1992). Studies (Hwa and Bevan, 1986) on myogenic tone in rabbit ear resistance arteries showed that ring segments of these arteries developed a maintained tonic contraction in response to mechanical stretch. The extent of the tone was dependent on the level of the applied stretch and the extracellular calcium concentration and it was not affected by the mechanical removal of the endothelium. Kulik *et al.* (1988) studied stretch-induced contraction in pulmonary arteries and illustrated a myogenic response in small ($<1,000\mu\text{m}$) feline pulmonary arteries using the myograph technique. It was also demonstrated that this myogenic response was not affected by the mechanical removal of endothelium. Kuo *et al.* (1990a), investigated the myogenic response in isolated porcine coronary arterioles and tested if an intact, functional endothelium was required for this response. This study was conducted under the no-flow condition in which the transmural pressure was altered by changing the height of the reservoir connected to the tested vessel. They found that elevation of intramural pressure evoked a pressure dependent constriction over pressure range of 40-140 cmH_2O and this response was not altered after mechanical removal of endothelial

cells.

Explanations of the controversies regarding the endothelium dependency in myogenic response include tissue and species variations, differences in vessel size, and especially, the differences in methodological procedures. For example, the blood vessels used by Rubanyi (1988) and Katusic *et al.* (1987) required exogenous vasoconstrictors to develop initial tone, whereas the flow rates and pressure steps were far outside the physiological range for that particular type of vessel. Nevertheless, the possibility that endothelial cells interact with the myogenic response by releasing vasoactive factors upon the stimuli of stretch could not be excluded (Rubanyi, 1988; Harder *et al.*, 1989).

Endothelium-Dependent Response

The discovery that endothelial cells (EC) play a pivotal role in the relaxation evoked by acetylcholine (Ach) in isolated rabbit aorta (Furchgott and Zawadzki, 1980) has initiated a true revolution in the world of cardiovascular sciences. It has become impossible to envisage the local regulation of blood flow, whether due to changes in physical conditions, to circulating vasoactive hormones, or to platelet products, without implicating endothelium-dependent changes in blood vessel diameter, thus resistance and flow. Indeed, the vascular endothelium is much more than just a lining for blood vessels. It plays an active role in a variety of physiological functions including maintenance of the fluidity of the blood, modulation of the tone of underlying vascular smooth muscle, inflammatory and immunological processes, etc. Studies in the past 10-15 years have revealed that the endothelium modulates the vascular smooth muscle tone by releasing a host of active substances including Endothelium Derived Relaxing Factors (EDRFs), prostacyclin, Endothelium Derived Hyperpolarizing Factor (EDHF) and Endothelium

Derived Contracting Factors (EDCFs) (Rubanyi, 1991; Gryglewski *et al.*, 1988; Furchgott, 1984). In addition, endothelial cells also serve as unique mechanoreceptors, sensing and transducing physical stimuli (e.g. shear forces, pressure) into changes in vascular tone by release of EDRFs or EDCFs (Rubanyi *et al.*, 1986; Koller and Kaley, 1990a; Kaiser *et al.*, 1986). These novel features of the endothelium opened an exciting study area for many scientists e.g. physiologists, pharmacologists, cell and molecular biologists and cardiologists.

Discovery of the endothelium-derived relaxing factor

Furchgott and Zawadzki (1980) first demonstrated, using the isolated rabbit thoracic aorta, that Ach-induced vasodilation was strictly endothelial dependent. This was concluded from studies where rubbing of the intimal surface, but not the adventitial surface, removed the capacity of aortic preparations to relax in response to Ach. They postulated that Ach acting on a muscarinic receptor in endothelial cells stimulates the release of a relaxing substance which in turn acts on the smooth muscle cells in the media to activate relaxation. This hypothesis was further proved by the "sandwich" method in which Ach-induced dilation was restored in an endothelial denuded arterial strip after it was mounted together with an endothelial intact strip in a "sandwich" arrangement with their intimal surfaces apposed (Furchgott, 1983). Although the released relaxing substance (later termed as Endothelium-Derived Relaxing Factor or EDRF) was not identified at that time, the possibilities for this substance being prostacyclin or another product of cyclooxygenase activity were ruled out since the inhibitor of that enzyme, indomethacin (40 μ M) and aspirin (100 μ M) had no effect on the relaxing action of Ach.

Several years later, Palmer *et al.* (1987) declared that Nitric Oxide (NO) release

accounts for the biological activity of endothelium-derived relaxing factor and this was the first convincing evidence for the identity of EDRF as NO. Using cultured porcine aortic endothelial cells on microcarrier beads as the donor of EDRF and strips of rabbit aorta in cascade for bioassay, not only did they show that infused NO and EDRF released by bradykinin possess identical characteristics in various tests, but also that the amount of NO (as determined by a chemiluminescence method) released by bradykinin could quantitatively account for the relaxation of the bioassay strip produced by the released EDRF. Similarly, Kelm *et al.* (1988) using another method for measuring NO, based on the reaction between NO and oxyhemoglobin to form methemoglobin, concluded that NO could account quantitatively for the relaxing activity of the EDRF released by bradykinin or ATP from cultured cells. Although the identity of EDRF and NO are still challenged by several laboratories, NO has been accepted widely as one of the EDRF(s) released upon the activation by various vasoactive substances including Ach, bradykinin, histamine, 5-hydroxytryptamine, adenine nucleotides, thrombin, noradrenaline, arachidonic acid and leukotrienes (Botting and Vane, 1990).

The precursor and the enzyme, as well as the co-factors required for the release of NO, soon became the focus of many studies. Shortly after the discovery of the identity of EDRF as NO, Palmer *et al.* (1988) first demonstrated the biochemical pathway for the synthesis of NO from the amino acid L-arginine. Using a chemiluminescence bioassay or mass spectrometry method to detect nitric oxide released from the cultured porcine aortic endothelial cells, they found that infusions of L-arginine and L-citrulline, but not D-arginine or other close structural analogues, reversibly enhanced Bradykinin-induced NO release. Mass spectrometry studies using ^{15}N -labelled L-arginine indicated that this

enhancement was due to the formation of NO from the terminal guanidino nitrogen atom(s) of L-arginine. These results suggest that L-arginine is the endogenous substrate for the generation of NO in vascular endothelial cells. Following this important discovery, many L-arginine analogues, such as N^G-Monomethyl L-Arginine (L-NMMA), N^G-Nitro-Arginine (L-NNA), N^G-Amino-L-Arginine (L-NAA), N^w-Nitro Arginine Methyl Ester (L-NAME), were reported to be potent inhibitors of NO synthesis. Johns *et al.* (1990) tested the inhibitory effect of L-NMMA on bradykinin or ATP-induced EDRF release and reported that L-NMMA (30-300µM) inhibited EDRF production in a dose-dependent manner and its action was specific to endothelial cells. Vargas *et al.* (1991) compared the inhibitory potencies of three different L-arginine analogues on EDRF function in the rat using both *in vitro* and *in vivo* preparations and illustrated that L-NNA is 70 times more potent than L-NMMA as an inhibitor of EDRF release from bovine aortic endothelial cells; thus elucidating a useful reagent for the investigations in this field.

Besides arginine analogues, superoxide anions and hydroquinone, a free radical scavenger, also were found to be inhibitors of EDRF/NO (Gryglewski *et al.*, 1986; Kaley *et al.*, 1989). Rubanyi and Vanhoutte (1986b) presented data illustrating that superoxide dismutase augmented the endothelium-dependent relaxation to Ach and doubled the half life of endothelium-derived relaxing factor(s). Therefore, they concluded that superoxide anions inactivate the relaxing factor(s) released by Ach from endothelial cells. Results of Gryglewski *et al.* (1986) supported this conclusion indicating that superoxide anions contributes significantly to the instability of EDRF. These results provide interpretations about how EDRF was inactivated as soon as being released, and therefore has such a

short half life (~30 seconds) (Palmer *et al.*, 1987).

While exploring the precursor of NO, Palmer and co-workers noticed that the strict structural and isomeric substrate specificity implicated the involvement of an enzyme in the generation of NO from L-arginine. Further investigation of the biochemical pathway for EDRF formation by Palmer and Moncada (1989) uncovered a novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. They found that the formation of [³H]citrulline was not observed unless [³H]arginine was incubated with both endothelial homogenate (the donor of the enzyme) and NADPH (1.5mM). Furthermore, [³H]citrulline formation from [³H]arginine was enhanced when NADPH, but not NADP, NADH or NAD (all at 1.5mM), was incubated with the homogenate, indicating NADPH is the specific cofactor of this enzyme. The enzyme was located in the 105,000 g supernatant of the homogenate, with no detectable activity in the washout pellet, showing that it is either a soluble enzyme or that it is only loosely associated with membrane. Also, they showed that there is a divalent cation requirement for enzyme activity. This enzyme was termed nitric oxide synthase (NOS) and the required divalent cation was most likely Ca²⁺, since the purified enzyme has an absolute requirement for calmodulin (Bredt and Snyder, 1990).

Cellular mechanism of the production of EDRF

Cellular mechanisms controlling the production of endothelial derived factors are not very clear; however, some studies have postulated certain intracellular pathways for the release of endothelial autacoids. First of all, many studies provide evidence supporting the idea that the release of EDRF or endothelial-dependent relaxation is

triggered by an increase in intracellular free calcium and that extracellular calcium is essential for this reaction. Lückhoff and Busse (1986) investigated changes in intracellular calcium using quin 2, a fluorescent calcium indicator, after stimulating cultured aortic endothelial cells with adenine nucleotides. They found that stimulation by adenine nucleotides (ATP, ADP and AMP) produces a dose-dependent increase in intracellular calcium. In the absence of extracellular calcium, ATP still raised intracellular calcium although endothelial responsiveness declined after repetitive stimulation, which indicated calcium mobilization from intracellular sources. Ryan *et al.* (1990) also studied the effect of bradykinin, ATP and thrombin on the concentration of intracellular calcium in bovine pulmonary artery endothelial cells using the fluorescent calcium indicator, Indo-1. They demonstrated a dual-phase intracellular calcium response. This was interpreted as an initial mobilization from intracellular stores rapidly reaching peak levels and lasting a few seconds. In the presence of extracellular calcium, the elevation maintained above baseline is interpreted as entry of calcium via plasma membrane channels. Griffith *et al.* (1986) studied the production of EDRF from rabbit aortic endothelial cells using an endothelial intact aortic strip as a donor, and an endothelial denuded aortic strip as an effector. They demonstrated that Ach provokes dilation in the effector strip only in the presence of extracellular calcium and this dilation was inhibited by agents that inhibit mitochondrial electron transport of F_1 -ATPase which uncouple oxidative phosphorylation. They therefore concluded that acetylcholine-induced EDRF release appeared to be dependent on both oxidative phosphorylation and extracellular calcium. Lückhoff and Busse (1990) provided evidences that hyperpolarization induced by initial rise in intracellular calcium, mobilized from

intracellular stores in response to receptor-binding agonists, was the driving force for transmembrane Ca^{2+} influx into endothelial cells; therefore, the formation of EDRF is controlled by the membrane potential.

Secondly, many studies also addressed other intracellular events that involved in the release of endothelial autacoids. Flavahan *et al.* (1989) demonstrated that in porcine coronary arteries, the endothelium-dependent responses evoked by serotonin, leukotriene C_4 or by the α_2 adrenergic agonist UK 14,304 were virtually abolished by pertussis toxin implying a linkage role of pertussis toxin sensitive G protein between activation of receptors and the release of EDRF. Derian and Moskowitz (1986) and Piroton *et al.* (1987) reported that in aortic endothelial cells, bradykinin and ADP activate the endothelium by increasing phosphoinositide hydrolysis; this illustrated the earlier event that triggers the release of Ca^{2+} from the intracellular stores after the receptor activation. Weinheimer *et al.* (1986), Lewis and Henderson (1987) illustrated that activation of protein kinase C by phorbol ester can inhibit endothelium-dependent relaxations, indicating a negative regulatory role of protein kinase C on EDRF release. Other studies, however, questioned the role of protein kinase C in the negative feedback control, although they demonstrated that activators and/or inhibitors of this enzyme may alter the responsiveness of endothelial cells to receptor-dependent agonists including bradykinin (Hecker *et al.* 1993).

Based on these studies, Busse *et al.* (1989) postulated a comprehensive summary of the intracellular mechanism for the release of EDRF. The hypothesis is illustrated in Figure 1. An agonist (e.g. Ach or bradykinin) binds to a membrane receptor which is coupled, via a GTP-binding protein (G-protein), to phospholipase C (PLC). This enzyme

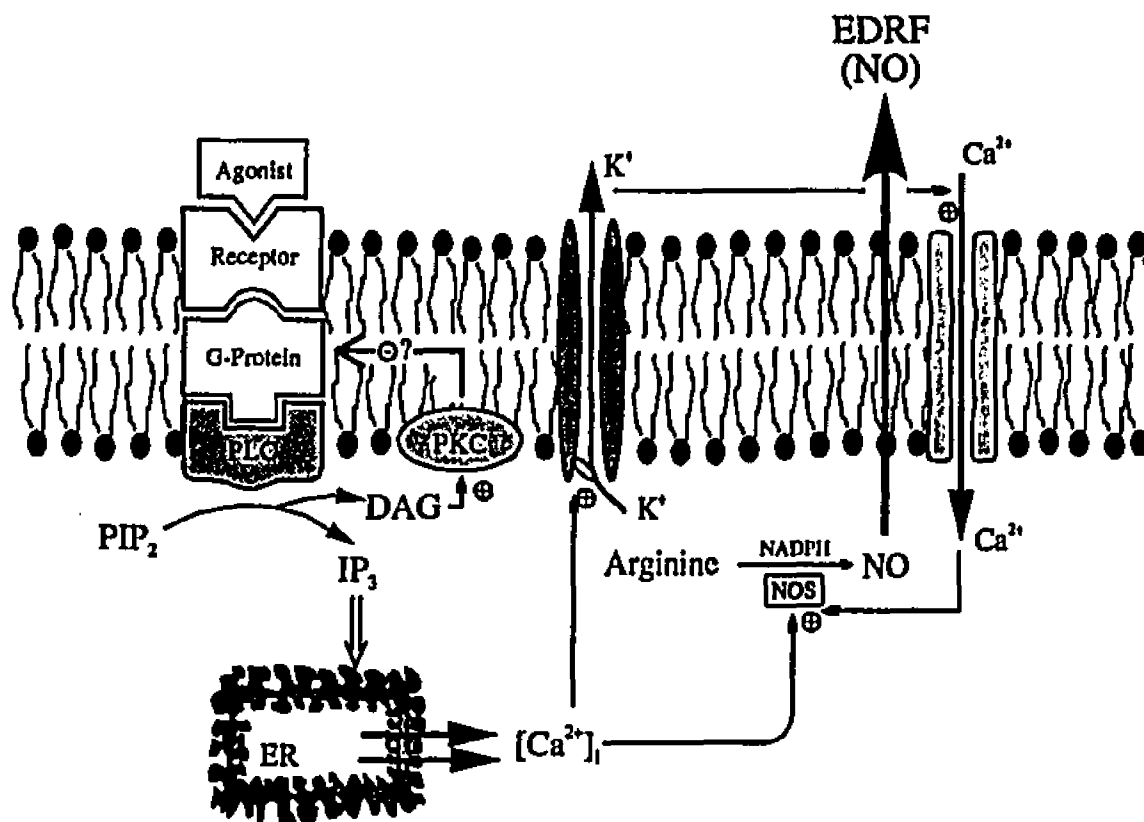


Figure 1. Schematic illustration of the intracellular mechanism for the release of EDRF/NO from L-Arginine by the endothelium. PLC: phospholipase C, PKC: protein kinase C, DAG: diacylglycerol, PIP₂: phosphatidylinositol-4,5-bisphosphate, IP₃: inositol-1,4,5-trisphosphate, NOS: nitric oxide synthase, ER: endoplasmic reticulum. ⊕: stimulatory influence, ⊖: inhibitory influence. See text for details

catalyzes the formation of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol-4,5-bisphosphate (PIP_2). IP_3 mobilizes Ca^{2+} from intracellular stores (endoplasmic reticulum, ER) thus increasing the intracellular free calcium concentration. This in turn activates Ca^{2+} -dependent K^+ -channels which induce hyperpolarization and facilitate transmembrane Ca^{2+} influx through as yet undefined channels. Further $[Ca^{2+}]_i$ elevation turns on the Nitric Oxide Synthase (NOS) which induces the formation of EDRF (NO) from L-arginine in the presence of NADPH. A possible feed-back control mechanism attenuating the agonist-induced response is provided by DAG which stimulates protein kinase C (PKC). The activation of PKC may phosphorylate the G-protein thereby interrupting the coupling between membrane receptors and PLC.

One more point in the production of EDRF which should be addressed is that the muscarinic receptor involved in this procedure appears to be a specific subtype, M_2 (Rubanyi, 1991). The activation of other muscarinic receptor subtype (M_1) will trigger the release of EDHF causing a transient smooth muscle relaxation by activating a calcium dependent K^+ -channel (Rubanyi *et al.* 1987); this response is different from the sustained relaxation produced by EDRF.

Transduction pathway for EDRF-mediated dilation

How does EDRF induce vascular smooth muscle relaxation upon its release from the endothelium? Accumulated evidence illustrates that the relaxation of smooth muscle cells caused by EDRF is due to activation of soluble guanylate cyclase and subsequent increases in the production of cyclic 3', 5'-guanosine monophosphate (cGMP).

Förstermann *et al.* (1986) studied the direct role of EDRF on the activation of soluble

gunylate cyclase. They injected the purified soluble guanylate cyclase, together with its substrate, into the lumen of vascular segments from rabbit aorta and canine femoral arteries for intraluminal incubation of the enzyme. They found that in endothelium-intact vascular segments the activity of gunylate cyclase was enhanced over values obtained by incubation in test tubes. Moreover, the stimulation was increased further by Ach in concentrations which cause relaxation of the vascular segments. Inhibitors (e.g. atropine, mepacrine, or nordihydroguaiaretic acid) which inhibit Ach-induced, endothelium-dependent relaxations also inhibited Ach-induced endothelium-dependent activation of gunylate cyclase. These results provided direct evidence supporting the hypothesis that Ach-induced EDRF increases cGMP levels of vascular smooth muscle by stimulation of a soluble guanylate cyclase. Pohl and Busse (1989) used radioimmunoassay to demonstrate that EDRF released by Ach (1mM) significantly increased cGMP level in platelets during its passage through the coronary vascular bed; while, treatment with the EDRF inhibitor hemoglobin completely abolished this cGMP elevation. Griffith *et al.* (1985) devised a bioassay system in which a rabbit coronary artery was perfused in series with an intact aortic strip. Their results showed that EDRF-induced relaxation is mediated by elevation of smooth muscle cGMP levels. Some evidence came from the inhibitory effect of methylene blue, an inhibitor of the activation of soluble guanylate cyclase (Katsuki *et al.* 1977). Since cGMP is the second messenger of the EDRF-mediated response, anything interrupting its production, e.g. methylene blue or hemoglobin, inhibitors of guanylate cyclase, will profoundly affect the subsequent responses. In fact, many studies have indicated that methylene blue or hemoglobin significantly inhibited EDRF-induced cGMP accumulation, or the

endothelial dependent relaxation (Marczin *et al.*, 1992; Martin *et al.*, 1985; Watanabe *et al.*, 1988).

Mechanism of cGMP-induced smooth muscle relaxation

Once cGMP level is elevated in the smooth muscle cells, it triggers another series of intracellular events which leads to the relaxation of smooth muscle cells. Rashatwar *et al.* (1987), Cornwell and Lincoln (1989) studied the mechanism by which cGMP relaxes smooth muscle cells and found that 1) increases in cGMP levels lead to decreases in intracellular Ca^{2+} concentrations evoked by contractile stimuli such as depolarizing concentration of K^+ or agonists (e.g. vasopressin), and 2) that cGMP-dependent protein kinase is the cGMP receptor protein that is both necessary and sufficient to account for the decreases in intracellular Ca^{2+} . The action site of cGMP-dependent kinase was studied by Raeymaekers *et al.* (1988) and Sarcevic *et al.* (1989) and it was reported that cGMP kinase catalyzes phosphorylation of phospholamban, a regulator of sarcoplasmic reticulum Ca^{2+} -ATPase, suggesting that the action of cGMP-dependent kinase is to regulate sarcoplasmic reticulum Ca^{2+} uptake. This conclusion also is supported by the study of Twort and Van Breemen (1988) showing that cGMP enhances Ca^{2+} uptake in 'detergent-skinned' smooth muscle cells in culture and prevents inositol-trisphosphate-induced release of Ca^{2+} in smooth muscle cells. According to these studies, Lincoln and Cornwell (1991) summarized the intracellular mechanism for cGMP evoked smooth muscle relaxation and proposed that when cGMP level was elevated by EDRF (NO), cGMP-dependent kinase was activated and it may, in turn, cause the phosphorylation of a Ca^{2+} -ATPase regulator protein, phospholamban. This will result in the activation of Ca^{2+} -ATPase and thus decrease intracellular calcium by enhancing the Ca^{2+} uptake into the

sarcoplasmic reticulum and/or preventing Ca^{2+} release from the intracellular stores. The decreases in intracellular calcium then leads to the relaxation.

Role of the endothelium as a mechanical sensor

In addition to the wide range of endogenous substances that bind to specific endothelial cell surface receptors, changes in flow, pressure and oxygenation also can stimulate the release of EDRF (as well as other endothelial substances). Indeed, by virtue of their position at the luminal surface of blood vessels, endothelial cells are situated ideally to act as sensors and modulators of such hemodynamic changes. It has been known for many years that an increase in blood flow through an artery elicits a dilation of the artery. More recently, the endothelium-dependent nature of this response has been shown by several groups (Holtz *et al.* 1984, Smiesko *et al.*, 1985). Furthermore, the flow-induced dilation has been shown to occur in isolated coronary arterioles (Kuo *et al.*, 1990b) and it has been demonstrated also to be endothelium-dependent at the microvasculature level in rat cremaster muscle (Koller and Kaley, 1990a). In perfused rabbit ear arteries *in situ*, Griffith *et al.* (1988) showed that flow-induced, endothelium-dependent vasodilation was greatest in those vessels in which shear forces were the greatest; indicating a linkage between shear stress and the release of EDRF.

The mechanism by which endothelial cells perceive and regulate changes in blood flow has been studied in large conduit arteries. Rubanyi *et al.* (1986) studied the potential mediator(s) involved in flow-induced, endothelial-dependent vasodilation using intraluminally perfused canine femoral artery segments and reported that in addition to prostacyclin, alterations in flow trigger the release of another relaxing substance from the

vascular endothelium. This substance has characteristics similar to EDRF released by Ach implying the identity of the mediator produced by alteration of flow with EDRF. On the other hand, Kaiser *et al.* (1986) showed that part of the flow-induced endothelial-dependent vasodilation could be blocked by eicosatetraynoic acid implicating a lipooxygenase or cyclooxygenase metabolite of arachidonic acid. Hence, endothelial prostacyclin production also may be stimulated by flow and may act in concert with EDRF to elicit vasodilation. This conclusion was supported by the study of Koller and Kaley (1990b) showing that in rat skeletal muscle microcirculation, prostaglandins, rather than EDRF, are likely to mediate flow-induced dilation.

How the endothelium senses and responds to hemodynamic changes is not known. Both pulsatility and shear stress generated at the interface between blood and endothelial cells are components of flow and appear to be important in the generation of EDRF. Ando *et al.* (1988) demonstrated that exposure of endothelial monolayers to gradually increasing laminar shear stress resulted in similarly progressive increases in cytosolic calcium concentration. Lansman *et al.* (1987) suggested that specific calcium channels located on the endothelial cell surface may act as "mechanotransducers" by responding to shear stress and may be involved in the generation of EDRF. In addition, laminar shear stress also has been shown to activate a potassium selective, inward current causing hyperpolarization in endothelial cells (Olesen *et al.*, 1988). The resultant hyperpolarization could stimulate the local release of EDRF and consequently cause relaxation of the vascular smooth muscle. Furthermore, there are evidences showing that certain vasoactive substances, such as substance P, serotonin and ATP, are present in endothelial cells and may act as transducers of hemodynamic changes. For instance,

these substances may be released from endothelial cells by or during an increase in flow to act on specific endothelial cell surface receptors which stimulate the release of EDRF and endothelial dependent vasodilation (Ralevic and Burnstock, 1993).

Local Regulation of Blood Flow, A Signal Integrating System

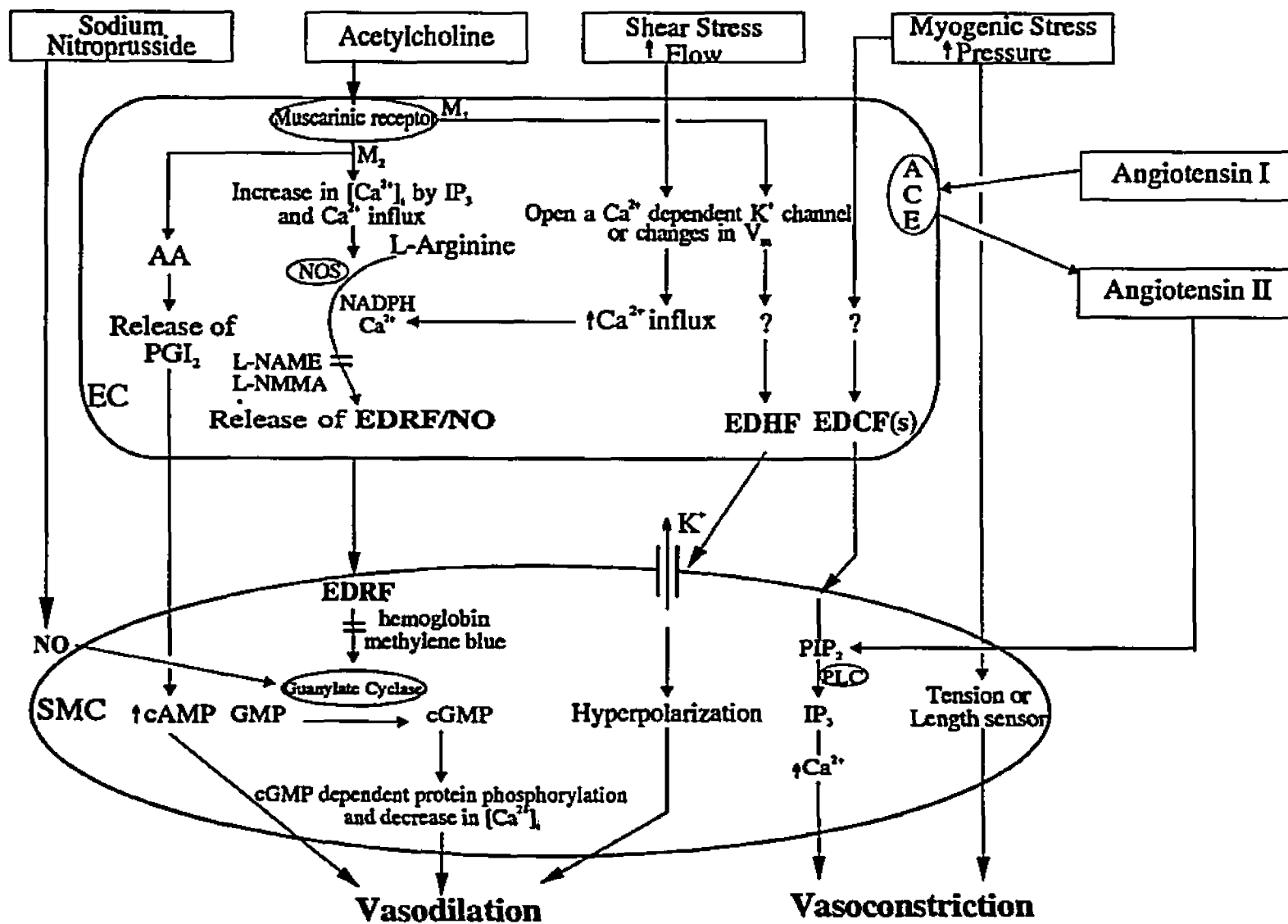
In summary, the regulation of vascular tone and the distribution of blood flow is governed by several mechanisms; whereas, humoral and neural controls are as important as the other mechanisms, they are not reviewed in detail in this chapter. Endogenous vasoactive substances include adenosine, bradykinin, substance P, acetylcholine, histamine, ATP, angiotensin(s), norepinephrine and many more. Numerous reactions evoked by these substances are mediated by endothelial cells; some agonists, however, act directly on smooth muscle cells. For instance, sodium nitroprusside and norepinephrine are endothelium-independent agonists and the responses to these substances are often used as a indicator of normal smooth muscle function. Other substances, such as angiotensin(s), maybe considered endothelial-independent agonists for their vasoconstrictor responses or endothelial-dependent agonists for the fact that Angiotensin II may stimulate a endothelial-dependent release of vasodilating prostaglandins from tissues in certain species (Ito *et al.*, 1991; Haberl *et al.*, 1990). In addition, angiotensin I (AI) is not biological active until being converted to a potent vasoconstrictor angiotensin II (AII) by angiotensin converting enzyme (ACE). Although AI is converted mostly in the lung, some investigators suggest that both renin and ACE are present in the walls of the peripheral vasculature and that the AII which participates in the regulation of vascular tone is generated locally (Oliver and Sciacca, 1984; Mizuno *et al.*, 1988; Swales and Thurston, 1973). Furthermore, since the vascular endothelial

cells are the major location of ACE (Kahr *et al.* 1991; Gohlke *et al.*, 1992), modulating local angiotensin conversion at different circulating levels may be another feature for the endothelium to have fine regulations on the vascular tone.

Figure 2 illustrates the summation of several cellular transduction pathways, discussed above, for vasodilation/constriction induced by agonists- or mechanical changes. The binding of Ach to m_2 subtype of muscarinic receptor(s) within endothelial cells (EC) leads to a elevation of intracellular calcium ($[Ca^{2+}]_i$) induced by both calcium influx and IP_3 formation. This results in the activation of nitric oxide synthase (NOS) and therefore elicits the release of EDRF (NO) from L-Arginine. Once released, EDRF diffuses into the underlying smooth muscle cells (SMC) and targets on the guanylate cyclase (Davies *et al.*, 1988). This in turn raises the cGMP level in SMC and leads to vasodilation through cGMP dependent protein phosphorylation. Notice that the release of EDRF was coupled with the production of prostacyclin (PGI_2) from the liberation of arachidonic acid (AA), while EDRF and PGI_2 act synergistically to evoke vasodilation (Nucci *et al.*, 1988; Pohl, 1993). The activation of m_1 receptor(s) may trigger the release of a hyperpolarizing factor (EDHF), through an unknown mechanism; this EDHF provokes SMC to hyperpolarize which leads to the relaxation. Increases in shear stress/or blood flow stimulates the EC to release EDRF by activation of NOS via the influx of Ca^{2+} , facilitated by hyperpolarization or opening of a Ca^{2+} dependent K^+ channel. The myogenic stimuli or the stretch produced by elevated pressure may directly stimulate SMC to contract through the tension or length sensor in SMC. Also, it may produce constriction by the production of endothelial contracting factor(s) (EDCF) via the cascade of the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2).

Reductions in shear stress/flow or intramural pressure, however, will oppose these responses by depressing the release of EDRF or EDCF or by changing the stretch or tension on the SMC (not shown). Angiotensin I is converted to angiotensin II by angiotensin converting enzyme (ACE) located on the surface of the EC; while, AII produces constriction through the hydrolysis of PIP_2 which was catalyzed by phospholipase C (PLC). Sodium nitroprusside is an endothelial-independent vasodilator acting directly on SMC through the generation of NO and it acts via the same transduction pathway as EDRF (activation of Guanylate cyclase), in the SMC, to elicit dilation. Interactions among these mechanisms depend on the specific local environment and the cellular activity of both endothelial cells and the smooth muscle cells.

Figure 2. Summarization of the cellular transduction pathways for agonist- or mechanical stimuli-induced vasodilation/vasoconstriction. See text for details.



CHAPTER 2

SPECIFIC AIMS AND THE SIGNIFICANCE OF THIS STUDY

Specific Aims

As reviewed above, the regulation of vascular tone and/or blood flow is a complex and involves several intracellular mechanisms and pathways. Since these mechanisms or pathways are series-arranged (from the endothelium to smooth muscle cells), interruption at any step (e.g. receptor binding, G-protein coupling, enzyme activating, factor releasing, ion channel opening etc.) will alter the normal sequences and yield biologically different effects. Furthermore, with various or specific combinations of these pathways/ mechanisms, vessels in different tissues or at distinct levels of the circulation may be regulated in dissimilar ways, i.e., in accord with their specific demands. Therefore, understanding the unique regulatory mechanism in different segments of the series-arranged arterioles is the central issue of this study. Our central hypothesis is that the local control of blood flow and autoregulatory behavior in the microcirculation is distinct at each level of the vasculature and is dependent on the cellular activity of the endothelium and its interaction with the local environment. The latter comprises the level of the vascular tone, the amount of stretch or shear stress on the mural cells, the specific intracellular messenger system, and the transduction pathway(s) for different intracellular signals. Hence, this study is designed to investigate the differential role of endothelial cells in governing both local control of blood flow, such as agonist-induced dilation/constriction of microvessels, and the flow/pressure- induced regulatory responses in different arteriolar segments of the

Intact microvascular bed.

Both *in vivo* and *ex vivo* pump-perfused preparations of the hamster cheek pouch microvasculature are utilized in this study to mimic the physiological environment and to establish a control of the regulatory driving forces, flow and pressure, and to control the humoral condition,

The specific aims are:

- 1) To test whether the microvessels in the hamster cheek pouch possess EDRF activity, and if so, to determine whether this EDRF activity is homogeneously distributed in different segments (specifically large, 2nd order, and small, 4th order, arterioles) of series-arranged vascular network.
- 2) To determine if local angiotensin conversion is accomplished via a pathway involving an endothelium-dependent mechanism which, in part, may be associated with an endothelial localized enzyme, angiotensin converting enzyme (ACE); and also, to compare the role of endothelial cells involved in local angiotensin conversion in large and small arterioles.
- 3) To investigate the autoregulatory behaviors initiated by increasing shear stress (flow) or elevating vascular stretch (pressure), in different arteriolar segments and to determine whether these behaviors are governed differently by the function of the endothelial cells in different segments of the series-arranged arterioles.

Significance of This Study

Most of the studies mentioned earlier in Chapter 1 were based on the data

obtained from *in vitro*, cultured cells or isolated conduit arteries; nevertheless, there were some studies dealing with the resistance-sized vessels in a variety of vascular beds. Rosenblum (1986) using an *in vivo* model demonstrated that relaxation of mouse pial arterioles to Ach or bradykinin is dependent on a normal endothelium. Furchgott *et al.* (1987) studied the perfused mesenteric arterial vasculature of the rabbit and reported that vasodilation by Ach was blocked almost completely after a 15 min perfusion of the vasculature with 0.2% collagenase, an enzyme capable of removing endothelial cells. Meanwhile, hemoglobin, an inhibitor of EDRF, also was found to markedly reduce the Ach response in the perfused mesenteric arterial vasculature of the rat. These results suggested that a major component of vasodilation of mesenteric resistance vessels in rabbit and rat by Ach is mediated by EDRF. Förstermann *et al.* (1987) and Pohl *et al.* (1987) investigated the resistance arterioles of the hindlimb of the rabbit *in vivo* and revealed that the EDRF mechanism is present in resistance arteries of a peripheral vascular bed. In addition, the EDRF-mediated arteriolar dilation also was illustrated *in vivo* in the skeletal muscle arterioles of rat cremaster (Koller *et al.*, 1989). These studies are the few cases that evidenced the EDRF activity in the level of microcirculation under *in vivo* or *in situ* conditions. Authors of these studies also pointed out that with those endothelial-dependent vasodilator agents, relaxation may be limited to certain species and/or blood vessels. In fact, Rubanyi and Vanhoutte (1988) discovered a heterogeneity of endothelium-dependent responses to Ach in canine femoral arteries and veins. Using a bioassay system, they demonstrated a depressed endothelium-dependent relaxation to Ach in femoral veins compared to arteries. They concluded that this is due to a masking effect of the direct stimulating action of Ach and decreased release of the same mediator

or the release of a different relaxing factor from venous endothelium. Other studies also illustrated that variations in endothelial function or in responsiveness of vascular smooth muscle cells may be responsible for the heterogeneity in endothelium-dependent responses to Ach between canine blood vessels of different anatomical origins (Vanhoutte and Miller, 1985). Interestingly, the heterogeneity of endothelium-dependent response to Ach also was found between distinct arteriolar segments in our previous studies; in which EDRF-activity was evident in downstream, 4th order arterioles but was absent in upstream, 2nd order arterioles in the hamster cheek pouch (Tang and Joyner, 1992). This finding of a heterogeneous distribution of EDRF activity creates an important issue: blood flow in the circulatory network may be regulated differently at different levels of series-arranged microvessels and these differential regulatory mechanisms may directly relate to the intra- or inter-cellular properties of the endothelium. This study has investigated these differential regulatory mechanisms in the series-arranged segments of arterioles in an intact, *in situ* (*in vivo* or *ex vivo*) vascular bed. Also it has addressed specifically the involvement of endothelial cells in these mechanisms.

In addition to mediating vasodilator responses, the endothelial cell also has been reported to modulate vasoconstrictor responses. Cocks and Angus (1984) studied vasoactivity in large coronary arteries and reported that removal of endothelial cells amplified the norepinephrine (NE) vasoconstrictor response. They postulated that the endothelium releases a vasodilatory substance upon activation of α_2 -adrenergic receptors and this substance antagonizes the vasoconstrictor response to NE. Removing the endothelium prevented the release of this substance; thus, the vasoconstrictor response to NE was enhanced. In smaller arteries or arterioles, however, it is not clear whether the

endothelial cell plays a role in modulating the NE response. Moreover, the vascular endothelial cell is also the site for the location of angiotensin converting enzyme (ACE); therefore, a functional endothelial cell may be important to the local conversion of angiotensin. To date, no data has been reported about the relation between endothelial function and the local production of angiotensin II, especially in series-arranged segments of the arterioles. This study has investigated the involvement of the endothelium in local angiotensin conversion and NE vasoconstrictor response in large and small arterioles; thus understood how reactions of these endogenous circulating hormones are modulated by endothelial cells in different microcirculatory levels.

It is mentioned earlier that endothelial cells act also as a mechanical sensor in response to alterations of shear stress. Koller and Kaley (1990a) reported a blood flow velocity-sensing phenomenon in rat cremaster muscle which suggested a new EC-dependent velocity-sensing mechanism for regulation of the blood flow in the microcirculation. They reported further that in the rat cremaster microvessels, the mediator generated by increase in flow is not EDRF/NO but rather prostaglandins (Koller and Kaley, 1990b). Recently, Kuo *et al.* (1991) studied isolated porcine coronary arterioles (40-80 μ m) and reported that in these coronary arterioles, flow-induced dilation was abolished by L-NAME or by mechanical removal of endothelium implying the involvement of endothelial cells and EDRF/NO pathway. Contradictory data, however, have been reported. Bevan and Joyce (1990) and Garcia-Roldan and Bevan (1990) demonstrated that in isolated rabbit ear arteries and isolated pial resistance arteries, the flow-induced responses were independent of the endothelium. They noticed that

increases in flow will produce either dilation or constriction. This response was dependent on the initial level of the vascular tone prior to altering the shear stress. Unfortunately, the preparation of isolated vessels could not maintain the physiological vascular tone; whereas, the influence of response in upstream arterioles on the downstream arterioles could not be investigated when the vessels were isolated from their natural vascular bed. This study has investigated flow-induced regulatory responses in an intact, *in situ* vascular bed in different segments of the arterioles and under different levels of intraluminal pressures. It will help to bridge the gap between information obtained from isolated vessels or cultured cells with that obtained from the whole organ studies.

By putting this information together we can imagine that this study will have a direct implication as the understanding of pathological conditions, such as hypertension (elevated vascular tone and impaired EDRF activity), diabetes and atherosclerosis (abnormal EDRF activity or impaired endothelial functions).

Finally, the hamster cheek pouch is a useful microcirculatory preparation with several unique advantages. The pouch is highly vascularized and all classes of microcirculatory vessels usually can be seen within the field of the microscope. Thus, it allows us to conduct the comparative studies on the various microvascular segments. In addition, the cheek pouch possesses both skeletal (retractor) muscle and cutaneous microcirculatory beds that make it particularly useful for comparative studies (Duling, 1973). Most importantly, the microvessels in the cheek pouch appear to lack adrenergic innervation (Joyner *et al.*, 1983) and the active vascular tone of the cheek pouch does not seem to be due to neural or adrenergic factors (Lombard, 1981); hence, the local control

(including myogenic and endothelial-dependent response) mechanisms are more important for blood flow regulation in this microvascular bed. Therefore, our data can be interpreted without considering the interference of the neural control mechanisms. Moreover, since the cheek pouch has been found to be an excellent site for allografting various tissues (Campbell *et al.*, 1979, Joyner *et al.*, 1992), circulation in certain vascular beds that can not be studied *in situ* (e.g. human retinal and choroidal tissue) could be studied by grafting into the cheek pouch; thus, the data obtained from this study will serve as important "control" for future studies investigating the local blood flow regulatory mechanisms of tissues grafted into the hamster cheek pouch.

By answering questions addressed in our specific aims, direct evidence of the role of endothelial cells involved in fine regulation of blood flow at distinct microcirculatory levels were provided. The preparations and protocols developed in this study initiated the investigation in intracellular pathway(s) implicated in the interaction of different regulatory mechanisms and cell to cell communications in regulating and distributing blood flow to tissues.

CHAPTER 3

MATERIALS AND METHODS

Materials and Animals

Intravital microscopy, the *in vivo* preparation of the hamster cheek pouch

Female Syrian golden hamsters (90-140g) were anesthetized with sodium pentobarbital (60mg/kg i.p.) and additional anesthetic (2 mg/100 g) was applied subcutaneously at 40-60 min intervals, or as needed. The right femoral artery was cannulated with polyethylene tubing (PE-10) to monitor the systemic blood pressure via a P23Db pressure transducer (Gould Statham Inc) connected to the catheter. Likewise, the right femoral vein was cannulated in the cases that test substances needed to be administered intravenously. The hamster then was placed on a perfusion board custom designed for microcirculatory observation, which was equipped with a small heating pad to maintain the hamster's body temperature. To prepare the cheek pouch for observation of the microcirculation, an incision was made in the skin of the hamster's right cheek through which the double layered cheek pouch was exposed. The cheek pouch was spread over a crystal located in the center of a perfusion chamber which is milled into the perfusion board. The cheek pouch was flattened and fixed into the chamber by pinning the outer edges to a rubber ring surrounding the crystal and it was suffused with warmed and aerated (5% CO₂, 95% N₂) Ringer's solution at constant flow rate of 2 ml/min. The temperature and pH of the superfusate were monitored and maintained at a constant level (35-37°C in temperature, 7.35-7.40 in pH). To aid visual clarity, the avascular connective tissue covering the surface of the cheek pouch was removed carefully under a

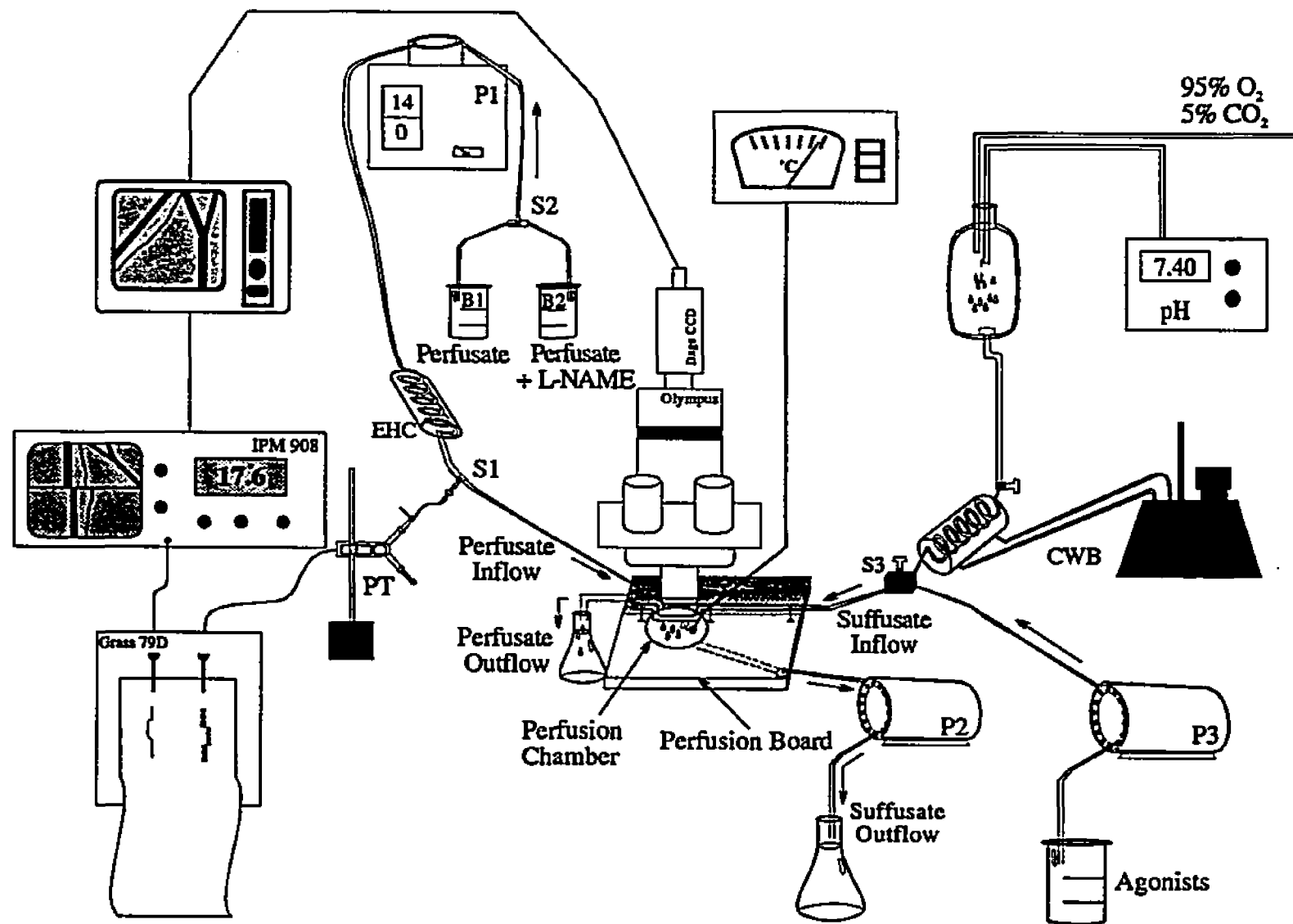
dissecting microscope. Illumination of the cheek pouch was provided by an Olympus 100 watt halogen lamp and the microvessels were observed via transillumination through an Olympus BH-2 microscope with an Olympus DPlan 10x objective. Visual display of the cheek pouch microvascular network also was provided by a TV monitor via a MTI CCD72 TV camera (Dage MTI, Michigan). For fluorescent microscopy, a mercury light source (filtered wavelength 490-510 nm) was used for the epi-illumination.

Preparation of the *ex vivo*, pump-perfused cheek pouch

After the hamster was anesthetized, the left common carotid artery was cannulated with polyethylene tubing (PE-50). To limit the perfused tissue primarily to the cheek pouch, a branch of the internal carotid artery was ligated. A peristaltic pump and a pressure transducer were connected via a 4-way stopcock to the catheter; thus, the perfusate can be pumped into the cannula while monitoring the perfusing pressure. The cheek pouch was prepared as described above and was suffused with the same Ringer's solution used in the *in vivo* study at a constant flow of 2 ml/min during all procedures. Unlike the *in vivo* study, the suffusate was aerated with 95% O₂, 5% CO₂ to oxygenate and to balance the pH. A thermal probe was placed in the chamber to monitor the temperature of the suffusate. After the cheek pouch preparation was completed, the right common carotid artery was tied so that any potential collateral blood supply to the cheek pouch was removed. As soon as the perfusion began, the right atrium of the hamster was exposed and opened for draining the perfusion solution out of the vasculature, thus maintaining a low venous pressure for fluid return.

A schematic for this *ex vivo* microscopic system is shown in Figure 3. To alter and control the perfusion flow in the microvessels, the cheek pouch was perfused

Figure 3. Illustration of the ex vivo system used in pump-perfused preparation of the hamster cheek pouch. See text for details



externally through a catheter (PE-50) in the left common carotid artery (perfusate inflow). A peristaltic pump (P1) and a pressure transducer (PT) were connected, via a 4-way stopcock (S1), to the catheter; therefore, the perfusion solution can be pumped into the vasculature via the catheter at desired rate (controlled by P1) while continuously recording perfusion pressure, via the output of the pressure transducer, onto the chart recorder (Grass 79D). In addition, an electrical auto-controlled heating coil (EHC) was placed in the perfusing-line to maintain a constant perfusate temperature ($\sim 35^{\circ}\text{C}$). Furthermore, the perfusing solution can be switched from beaker 1 (B1) to beaker 2 (B2) by switch 2 (S2) as needed. Outflow of the perfusate drained from the right atrium of the hamster flowed into the recessed area of the perfusion board (darker area in Fig.3) and was collected (perfusion outflow).

In the right-hand portion of the schematic, the suffusion line and its control are depicted. A circulating water bath (CWB) was used for maintenance of a constant suffusate temperature. The hamster was placed in the recessed area of the perfusion board facing towards the perfusion chamber where the cheek pouch is positioned. The suffusion solution flowed into suffusion line (suffusate inflow), a metal tube, which has outflow openings at the bottom of its concave shape and this part of the tube is positioned on the top of the cheek pouch inside the perfusion chamber. After flowing over to the cheek pouch, the suffusion solution was collected in a cylinder of trough through a channel built into the perfusion board, and it then was pumped out by pump 2 (P2). Pump 3 (P3) and switch 3 (S3) were used to facilitate the addition of testing substance(s) (e.g. Ach or sodium nitroprusside (SNP)) into the suffusion line. The microscopic image of the cheek pouch was fed via a TV camera into a monitor to provide visual display of

the microvasculature. An Image Shearing Monitor (IPM 908) was used to measure the diameter changes and its output can be traced continuously by the chart recorder (Grass 79D) (see later section for detail).

Calibration of the peristaltic pump

To obtain an accurate perfusion flow rate, the peristaltic pump used in the perfusing procedure (P1 in figure 3) was calibrated for the relation between the pump speed and the volume flow of the output. At the tip of the catheter, the output was collected in an unit of time (T) (e.g. 1 min) and its weight (W) was measured. Assuming the specific gravity (g) of the output solution is 1 gram/ml which is equal to that of the water, the volume flow rate (Q) then was calculated as follows: $Q=W/(g*T)$. Repeating this procedure at different pump speeds, a calibration curve was obtained as shown in Figure 4. The linear regression ($Y=0.003X+0.085$) of this pump speed/volume flow relation showed a high linearity ($r=0.999$); therefore, this regression equation was used to predict pump speeds that give rise to desired perfusion flow rates. The same calibration procedure was conducted for the pump 3 which was used for agonists application (P3, Fig. 3).

Arteriolar branching pattern

The arteriolar network was characterized according to its branching pattern and the vessel orders were classified based on the description of Wiedman (1968). As shown in Figure 5, the major artery, superior saccular (SS) artery in this case, entering the viewing area gave off branches designated as 1st order arteriole(s). Branches of the 1st order arterioles were considered 2nd order arterioles (A_2) and these vessels average

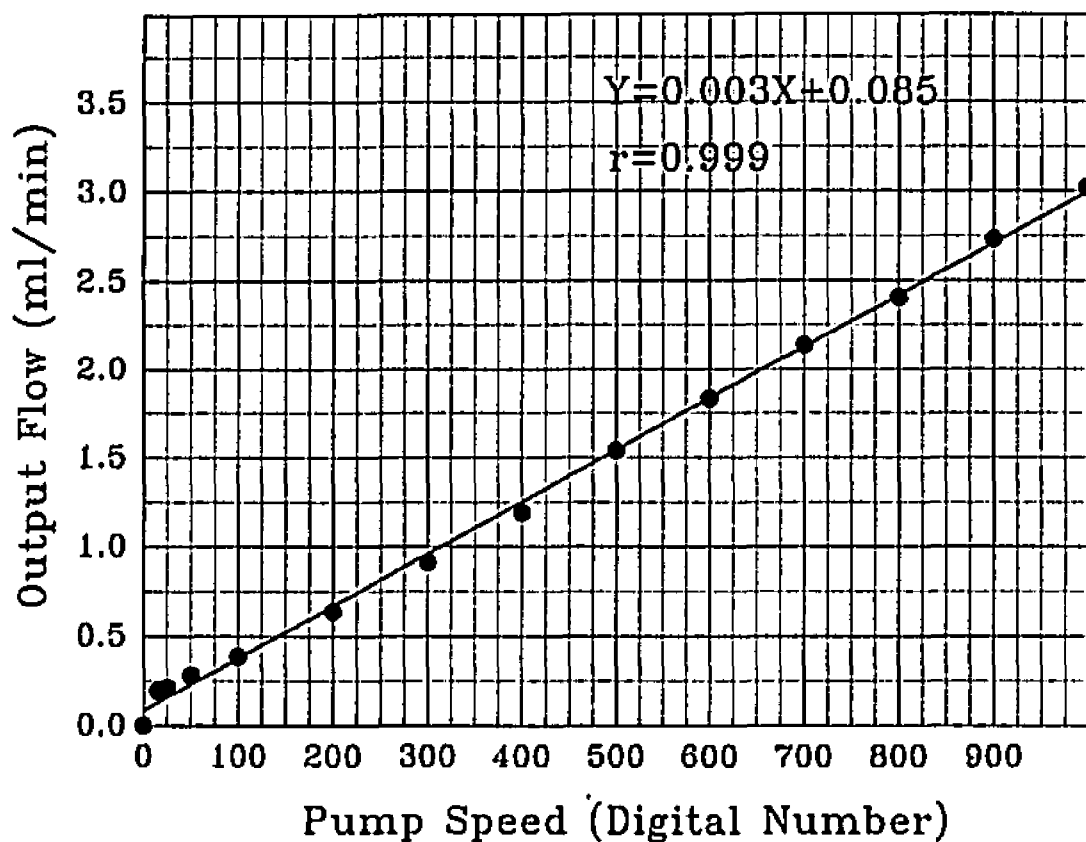


Figure 4. Calibration curve of the relation between output flow rate and pump speed used in the *ex vivo* preparation. The regressed relation has a high correlation coefficient; therefore, is used to predict the pump speeds which give rise to desired output flow rates of 1 ml/min, 2 ml/min, and 3 ml/min.

40–50 μm in diameter. Further branches were numbered consecutively until the capillaries were seen. The 4th order arterioles (A_4) became, in most cases, terminal arterioles and their size ranged from 7–20 μm in diameter. Segments of 2nd and 4th order arterioles were chosen and tested in this study and these arterioles were series-arranged in the vascular network as demonstrated in Figure 5.

Materials and chemicals

All chemicals used in this study were prepared freshly from frozen stock solutions and concentrations varied by dilution with isotonic saline (0.9%). One exception is the solution of fluorescein isothiocyanate (FITC)-dextran which was made using Ringer's solution without glucose as the solvent. Acetylcholine and sodium nitroprusside were purchased from Eastman and Elkins-Sinn Inc. respectively. The fluorescent microspheres used in centerline velocity measurement were purchased from Polysciences, Inc. and diluted in Ringer's or in the perfusion solution. L-NMMA and L-NAME were purchased from Calbiochem Inc., while other chemicals used in this study were products of Sigma Chemical Company.

Ringer's solution used for suffusion of the cheek pouch was composed of the following (in mM): 5.5 Glucose, 25.0 NaHCO_3 , 5.0 HEPES, 2.0 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 132.0 NaCl, 4.6 KCl, 1.2 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. Its pH is adjusted to 7.4 and balanced by aerating with mixed gas (5% CO_2 , 95% N_2 for *in vivo*, and 5% CO_2 , 95% O_2 for *ex vivo* preparation).

The perfusion solution used in the *ex vivo* preparation was composed of mammalian Ringer's (same as the suffusion solution) plus 1% dextran (162K, M. W.) and 0.25% bovine albumin with pH adjusted to 7.4. The osmolarity of this solution was between 280–290 (mOsm). At the beginning of the perfusion, 150 unit/100 ml heparin

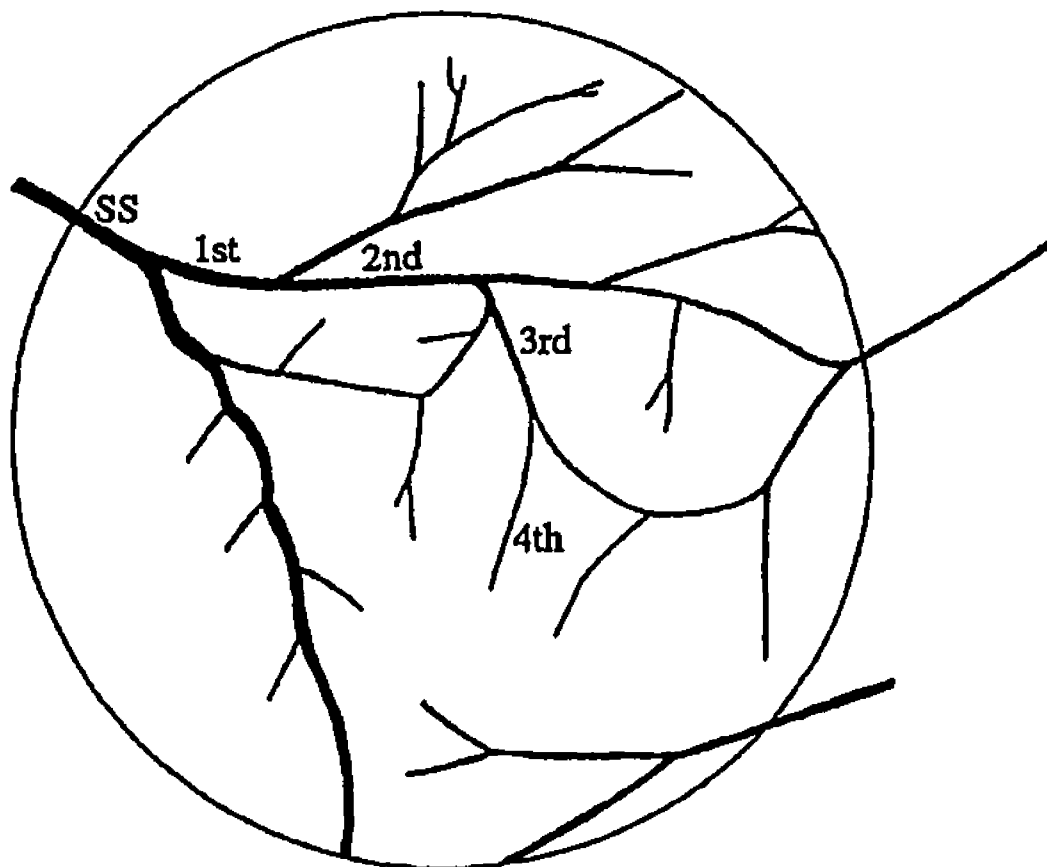


Figure 5. Branching pattern for arterioles, which are in the viewing area of the hamster cheek pouch, and vessel classification. SS: superior saccular artery. 1st: first branch from the SS which is classified as 1st order arteriole. 2nd: branch from 1st order arteriole (2nd order). 3rd and 4th are in turn branches from 2nd and 3rd order arterioles and they were classified as 3rd and 4th order arterioles.

was added to the 1st 100 ml perfusate to prevent the blood from clotting.

Measurement of Vasoactive Responses

Application of the testing agents

Glass micropipettes, which are pulled and beveled to a tip diameter of 10-16 μ m, were filled with a given concentration of the drug to be tested. The fluid delivery was controlled with an injection pump which delivers a constant volume (10 μ l/min for 1 min); thus, depending upon the concentration of the drug, a given amount was applied topically to selected microvessels. The vehicle (0.9% saline) was used as a control. The pipette was positioned, via a micromanipulator, directly on top of the selected vessel, hence the drug can be applied specifically to that segment of the vessel.

In experiments using *ex vivo* preparations, the tested agents were applied topically by adding into the suffusion-line via pump 3 and switch 3 (see Fig. 3). Therefore, these agents were mixed uniformly in the perfusion chamber.

Measurement of arteriolar diameter

The variation of the arteriolar diameter induced by the application of vasoactive substance(s) was used to determine the vasoactive response of that particular vessel. To measure the dynamic changes of the vascular diameter, an image shearing monitor was connected into the closed circuit TV system (see Fig. 3, IPM 908). This image shearing system used a cathode ray tube for visualization of the split image; this allowed rotation of the image in relation to the cutting line, which is always horizontal. For example, a vessel of interest can be rotated electronically and therefore be aligned perpendicular to the cutting line. A schematic is shown in Figure 6 demonstrating the principle of the

Figure 6. Schematic display of the principle of the image-shearing method. The video image of selected arteriole is rotated electrically (Image Rotating) thus aligning the interested segment of the arteriole perpendicularly to the cutting line (which is always horizontal). The selected segment of the arteriole then is split at the cutting line, a raster line that can be set freely along the Y axis (perpendicular to the cutting line). Once the inner surface of wall A in the bottom half of split image is sheared, i.e. placed, tangent to the inner surface of wall B shown in the top half of the split image, the displacement (D_s) by the shearing is equal to the inner diameter of this arteriole.

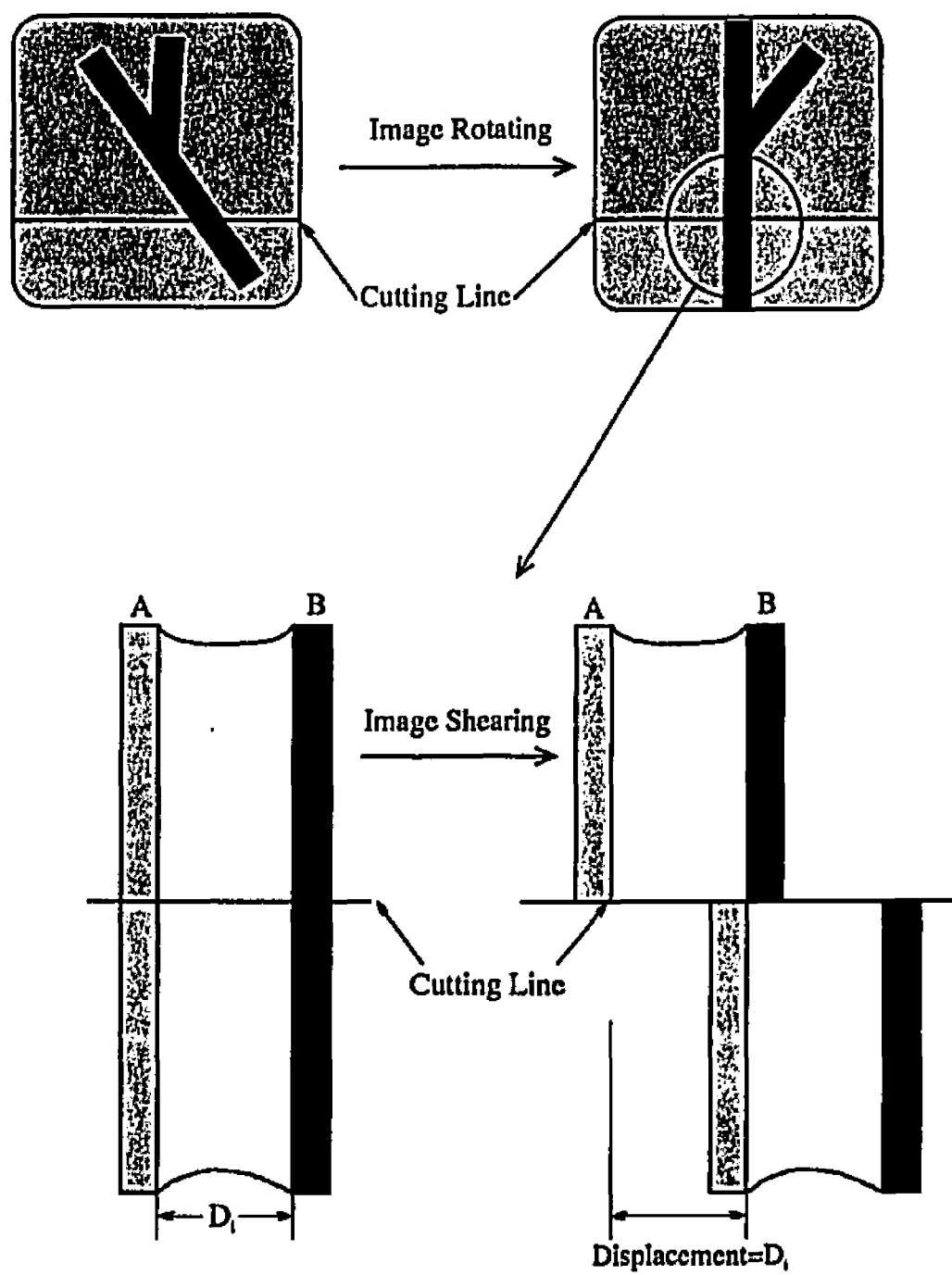


image shearing monitor. After aligning the selected vessel perpendicularly, a cutting line can be positioned (vertically) as desired. Thereafter, the bottom part of the image can be split/or sheared to a position where the inner surface of the wall A shown in the bottom half of the image was tangent to the inner surface of wall B shown in the top half of the image. Thus, a selected portion of the television image had been "sheared" and horizontally displaced by an amount D_i which is equal to the inner diameter of the tested vessel. This displacement was proportional to an adjustable voltage E, which then became a measure of horizontal dimension in a manner analogous to the micrometer adjustment setting (or calibrating) from a known dimension scale (e.g. standard micrometer slide). Accordingly, during the application of an agonist, as long as the inner surfaces of wall A and wall B are kept tangent to each other, the variation of the displacement voltage actually "traced" the diameter changes induced by the stimulation. Once calibrated, the digital meter of the image shearing monitor can display the result in micrometers. In addition, the signal output of DC current allowed continuous recording of the results onto the chart recorder. The accuracy of this system is of the order of 0.1 μm (Intaglietta and Tompkins, 1973).

Recording of vasoactive responses

Prior to any drug testing, basal/or control diameters (D_c) of large and small arterioles were recorded; thereafter, drug applications began. The time course of diameter changes was traced on a Grass 79D polygraph 1 min before (basal diameter recording), during (experimental diameter (D_E) recording) and 2-5 min after (vessel recovering) the drug application. There were 3-5 min intervals between each test. The response testing was basically in a sequence such that the downstream 4th order arterioles

were tested first followed by the upstream 2nd order arterioles. Agonists were tested in a random order with the exception that the tests for AI and AII were separated by testing other vasoactive agents to avoid tachyphylaxis.

Light-Dye treatment for endothelial impairment

Excitation wavelength light (EWL) illumination of fluorescence has been reported to impair the function of vascular endothelial cells (Povlishock *et al.*, 1983; Rosenblum *et al.*, 1987; Koller *et al.*, 1989). Therefore, the "light-dye" technique similar to that used by Koller *et al.* (1989) was applied in this study to impair endothelial function. The technique of Koller *et al.* was modified by using fluorescein isothiocyanate (FITC) labelled dextran (150,000 M.W.), rather than sodium fluorescein, as the "Dye" since these FITC tagged macromolecules remained within the vascular space for many hours following systemic administration unless macromolecular leakage was induced (Boric *et al.*, 1987). The "light" component of this technique was the illumination of a selected arteriole with EWL ($\lambda=490$ nm) for a certain period of time.

FITC-dextran was administered intravenously through the catheter in the femoral vein. The dose of FITC-dextran used in this study was 50 mg/100 g body weight according to the report of Svensjö and Joyner (1983). Before the EWL illumination, FITC-dextran was allowed to circulate for 5-10 min. Thereafter, the selected arteriole was subjected to light-dye treatment by exposing a small area (about 1 mm²) to EWL for epi-illumination.

Determination of the duration for EWL exposure

The duration of EWL exposure was determined under a fixed intensity of light

while recording the response to Ach in both A_2 and A_4 as an index for endothelial function. Meanwhile, the responses of A_2 and A_4 to sodium nitroprusside also were tested to assure normal smooth muscle function.

In the study of Koller *et al.*, the duration of a effective illumination for light-dye treatment was 100-140 seconds and this duration was long enough to impair the endothelial function in 50-100 μ m arterioles of skeletal muscle. Therefore, the duration of EWL illumination for the light-dye treatment in this study started from 2 min and it was gradually prolonged in steps (10 second each step) until the maximal loss of the Ach response was obtained.

Measurement of Centerline Velocities and Determination of Local Volume Flow

In the *ex vivo* preparation, fluorescent microspheres (2 μ m in diameter) were injected into the perfusion-line through S1, or added to the perfusate at a concentration of 0.001%. The centerline velocity of these microspheres was measured using a dual-window videoanalyzer. This videoanalyzer (IPM 204) has two windows which can be positioned over the selected vessel using the video image. An electrical output, which is proportional to the light intensity within the window, was obtained from each window. Therefore, each window can function as a photometer and it can follow variations in light level faithfully. Signals from these two windows were converted into electrical output DC voltages which can be recorded on a polygraph chart recorder (Grass 79D). Output signals from the videoanalyzer were illustrated in Figure 7. When a fluorescent microsphere passed through the selected arteriole inside the windows, spikes in the output signals were recorded from both windows. The time delay (t) between signals/spikes from the upstream and downstream windows can be obtained from the

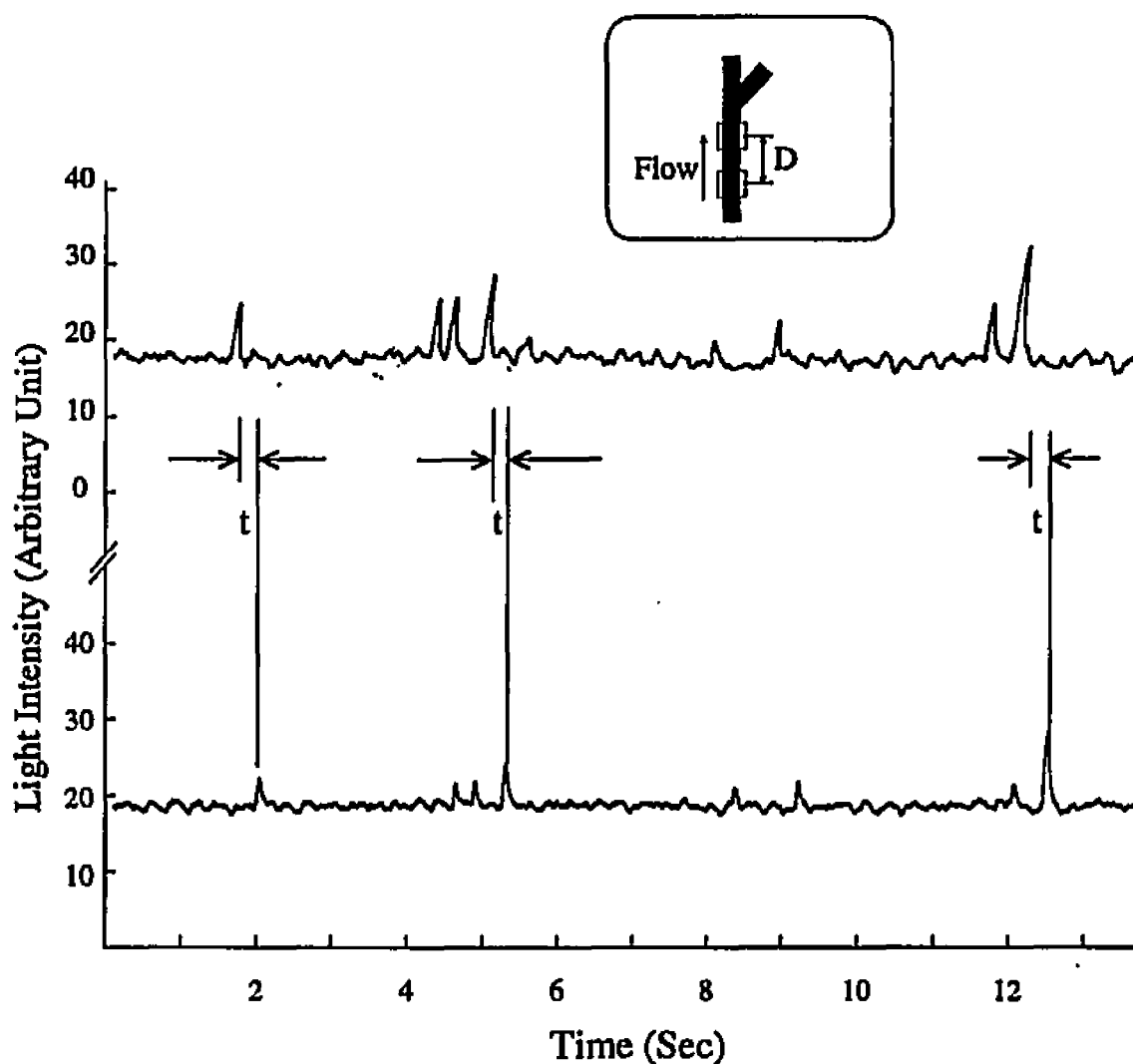


Figure 7. Time-dependent output from two windows of the videoanalyzer used for the measurement of the centerline velocity. The time delay (t) between signals captured from upstream (upper trace) and downstream (lower trace) windows can be obtained from the time scale of the chart. For example, the average t of three measurements illustrated in this figure is 0.28 sec. Using t and the distance (D) between the two windows, 213.5 μm in this case, the centerline velocity (V_c) can be calculated: $V_c = D/t = 0.76$ (mm/sec). The small illustration at top shows the alignment of the selected vessel and positions of the upstream and downstream windows. Arrow at left indicates the direction of the flow.

recorded chart. The distance, (D) in μm , between the two windows can be set. Thus, the centerline velocity for these microspheres was calculated by the equation: $V_c = D/t$. Using this centerline velocity and measurements of the vessel diameter ($d=2r$), the local volume flow (Q_m) can be calculated by: $Q_m = \pi r^2 V_m$, where $V_m = V_c/k$. The normalizing coefficient, k, is the factor compensating the velocity profile in the vessel and it varies with vessel size, e.g. 1.3-1.6 for smaller arterioles (Baker and Wayland, 1974).

For *in vivo* preparations, fluorescent microspheres were injected through a catheter in the femoral vein of the anesthetized hamster at a concentration of 5% (in Ringer's solution) for 1-2 ml. After 3-5 minutes stabilization, the small or large arterioles were selected. The *in vivo* microscopic image of a selected vessel was analyzed by the dual window videoanalyzer same as described above while the systemic blood pressure of the hamster was monitored through the pressure transducer connected with the catheter in the femoral artery.

Data Analysis

Responses of arterioles to vasoactive agents were expressed as percentage change in control diameter ($\% \Delta D_c$). This was actually the diameter change (ΔD) computed as a percentage of the control (or basal) diameter and it was calculated as follows:

$\% \Delta D_c = [(D_E - D_c)/D_c](100\%)$, where D_c is the control diameter measured before the application of the agent, and D_E is the experimental diameter, which is the maximum constricted or dilated diameter for each arteriole after the drug application. Therefore, if the tested arteriole dilated, D_E would be greater than D_c , thus yielding a positive $\% \Delta D_c$ value. Accordingly, when the tested arteriole constricted, $\% \Delta D_c$ should be a negative value.

In some experiments, the term of $\%D_c$, a normalized diameter expressing D_E as a percentage of D_c , was used to characterize the diameter changes; $\%D_c$ was calculated as follows: $\%D_c = (D_E/D_c)(100\%)$. It is apparent that a $\%D_c$ value greater than 100 indicates a dilation and less than 100 indicates a constriction.

Another term used in this study is the percent conversion for angiotensin I (AI) to angiotensin II (AII). This is indeed a ratio between the response of AI and the response of AII, and it is calculated by the following formula:

$$\text{Percent conversion (C\%)} = [(\% \Delta D_c \text{ of AI}) / (\% \Delta D_c \text{ of AII})](100\%).$$

This formula differed slightly from that proposed by Gerlings and Gilmore (1973). Since molar concentrations were used in this study, it is unnecessary to include the factor of 125% to take the differences between AI and AII gram concentrations into account.

All data were presented as means with standard errors (SE). Different statistical procedures were used including: two way analysis of variance, multiple comparison (Tukey test, and LSD test), linear regression, and student *t* (paired) test, depend on the specific experimental designs (Ott, 1988). P values less than 0.05 were considered significant.

CHAPTER 4
DIFFERENTIAL ROLE OF ENDOTHELIAL FUNCTION
ON VASODILATOR RESPONSES IN SERIES-ARRANGED ARTERIOLES

Introduction

The pioneering work of Furchgott and Zawadzki revealed that the relaxation of isolated preparations of arteries (rings, transverse strips, or helical strips) by acetylcholine was strictly dependent on the presence of endothelial cells on the intimal surface of the preparation (Furchgott and Zawadzki, 1980; Furchgott, 1984). This provided the *in vitro* evidence for the obligatory role of endothelial cells in mediating vasodilator response to acetylcholine. Years later, several studies demonstrated further, using an *in vivo* method, that acetylcholine-induced vasodilation is dependent on intact endothelial cells (Rosenblum, 1986; Furchgott *et al.*, 1987; Koller *et al.*, 1989). Now, it is clear that acetylcholine acts on the muscarinic receptor of the endothelial cells and stimulates the release of a substance termed endothelium-derived relaxing factor (EDRF), which then induces the relaxation of vascular smooth muscle cells by the activation of guanylate cyclase and the consequent elevation of the intracellular cyclic guanine monophosphate (cGMP) (Furchgott, 1984; Gryglewski *et al.*, 1988; Nakatsu and Diamond, 1988; Davies *et al.* 1988).

Several studies reported that EDRF was likely a free radical since its activity was inhibited by hydroquinone, a free radical scavenger, and it was degraded by superoxide anions (Kaley *et al.*, 1989; Rubanyi and Vanhoutte, 1986 a,b; Gryglewski *et al.*, 1986). Recently, it has been accepted widely that at least one of the EDRFs was identical with

nitric oxide (NO), which is released enzymatically from the guanidine group of L-arginine causing a sustained phase of vasodilation and this provides a unique pathway for the mechanism of acetylcholine-induced vasodilation (Palmer *et al.*, 1987; Rees *et al.*, 1989; Tschudi *et al.*, 1991; Rubanyi, 1991). Investigators also reported that unlike the various endothelium-dependent vasodilators (such as acetylcholine, ATP, bradykinin, histamine, A23187, substance P), the removal of endothelial cells did not interfere with relaxation produced by sodium nitroprusside, adenosine, and/or isoproterenol. Based on these facts, Rosenblum (1986) developed, and Koller *et al.* (1989) modified a light-dye technique that was presumed to impair the function of endothelial cells without damaging vascular smooth muscle cells. They demonstrated that in cerebral arterioles of mice and in cremaster arterioles of rats, acetylcholine-induced arteriolar vasodilation was inhibited by light-dye treatment, whereas the vasodilator response to sodium nitroprusside was not altered. These were the first studies to report EDRF-like activity in the microcirculation. However, segmental effects of this endothelial impairment on the vasodilator response in various series-arranged arterioles have not been studied.

Thus, this study addresses the following: do arterioles of the hamster cheek pouch contain EDRF-like activity, and if so, is this activity homogeneously distributed in the serially-arranged arterioles of the microcirculation?

Experimental Protocols and Design

Determination of the light-dye treatment time period on small (4th Order) and large (2nd Order) arterioles

According to the results of Koller *et al.* (1989), the initial period of time for EWL

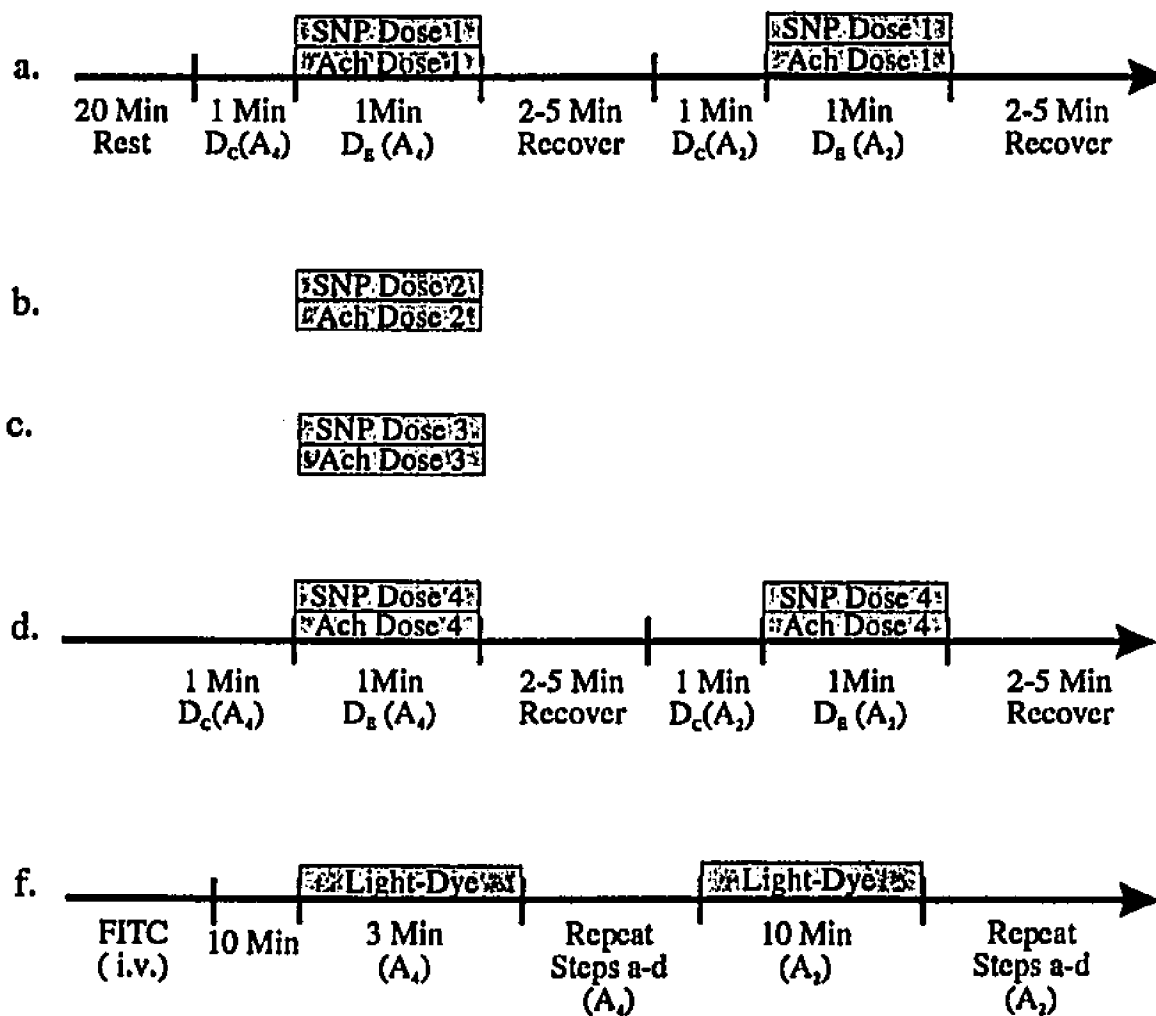
illumination was begun at 2 min in our intravital microscopic system for 4th order arterioles. This exposure time was gradually extended to 3 min at approximately 10 second intervals until the maximal loss of response to Ach was obtained. Based on these data, the time for exposure of 2nd order arterioles was begun at 3 min and it was prolonged gradually to 10 min at 1-3 min intervals. Since 10 min is an apparent period of time beyond which irreversible injury of microvessels occurs (Reed and Miller, 1988), longer periods of light-dye treatment were not tested. After 3 and/or 10 min of light-dye treatment, no platelet aggregation was observed in either 2nd or 4th order arterioles and these microvessels remained responsive to other vasoactive agents.

Effect of endothelial function on vasodilator responses in different segments of the series-arranged arterioles, as determined by impairment using light-dye treatment

Arterioles tested were series-arranged 2nd order (30-50 μ m) and 4th order (10-20 μ m) microvessels and they were separated by two bifurcations (Fig.5).

The time sequence of this protocol is illustrated in Figure 8. Various doses of Ach and SNP were tested in the control state (i.e., before light-dye treatment) on both 2nd and 4th order arterioles. The lowest dose of Ach and SNP (10^{-9} M) was tested first on a selected 4th order arteriole and then on the upstream 2nd order arteriole. Then, higher doses of Ach and SNP (10^{-7} , 10^{-6} , and 10^{-5} M) were tested on the same 4th and 2nd order arteriole in the same manner. FITC-dextran (150K) then was injected through the femoral vein cannula and allowed to circulate for 10 min and light exposure was applied to the site previously tested on 4th order arteriole for 3 min while continuously measuring the vessel diameter. The same dose-response testing of Ach and SNP on this 4th order arteriole was instituted. Subsequently, the area of the larger, 2nd order,

Figure 8. Experimental protocols for testing the light-dye effect on dose responses to acetylcholine (Ach) or sodium nitroprusside (SNP) in large, 2nd order and small, 4th order arterioles. The responses to Ach or SNP were tested in four doses: a) the lowest dose at 10^{-9} M, b) the second dose at 10^{-7} M, c) the third dose at 10^{-6} M, and d) the fourth dose at 10^{-5} M. At each dose, responses in the small arteriole (A_4) were tested first followed the test in the large arteriole (A_2), one agonist at a time. In step f, FITC-dextran was injected intravenously and light-dye treatment was first conducted in A_4 (3 minutes) followed by the test of the dose responses to Ach or SNP in the same manner described in steps a-d. Thereafter, 10 minutes light-dye treatment was instituted in A_2 followed by the dose responses test as described in steps a-d.



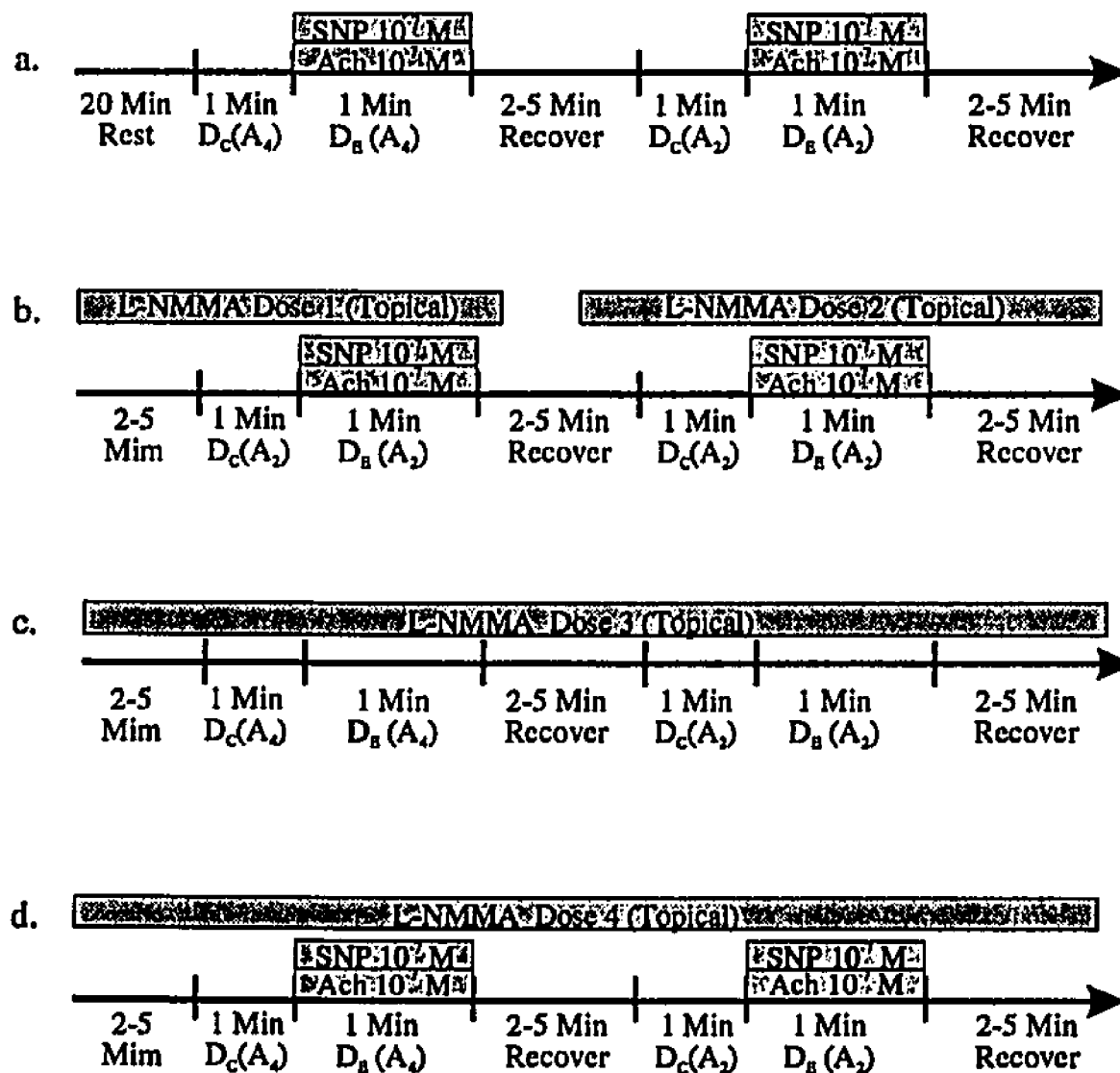
arteriole previously tested was found and exposed to light for 10 min. Then, dose response testing for Ach and SNP was repeated in the same manner as previously described. In some preparations, this order of exposure to light-dye treatment was reversed.

Role of EDRF-like activity in mediating Ach-induced vasodilator response in different segments of the series-arranged arterioles, as determined by L-NMMA, an inhibitor of EDRF/NO synthesis

In this series of experiment, L-NMMA was used to block the synthesis of EDRF/NO while stimulation with Ach was completed before and during L-NMMA application to test the EDRF-like activity in series-arranged hamster cheek pouch arterioles.

The protocol is illustrated in Figure 9. In the control state, vasodilatory responses to Ach and SNP (both at 10^{-7} M) were tested on both 2nd and 4th order arterioles. After this control recording, L-NMMA was added into the suffusion solution by pumping it into the suffusate line through a three-way stopcock (S3 in Fig. 3) at a constant speed of 0.2ml/min starting with the lowest concentration (1.5×10^{-6} M). Due to the dilution effect, the final concentration bathing the tissue was 1.5×10^{-7} M. Two minute after starting L-NMMA suffusion, responses of 2nd order arterioles to the same concentration of Ach and SNP were tested. Then, using a similar procedure, vasoactive testing was repeated, while increasing the concentration of L-NMMA in the suffusate to 1.5×10^{-6} , 1.5×10^{-5} , and 1.5×10^{-4} M. During the last two doses of L-NMMA, Ach and SNP responses also were tested on 4th order arterioles. Control diameter (D_0) was measured before and two min after the addition of any dose of L-NMMA without the application of Ach and SNP.

Figure 9. Experimental protocols for testing the effect of EDRF/NO blockade using N^G-monomethyl L-Arginine (L-NMMA) on acetylcholine (Ach) and sodium nitroprusside (SNP) responses in large (A₂) and small (A₄) arterioles. Control responses to Ach or SNP (both 10⁻⁷ M) were first tested in A₄ and then in A₂ (step a). Then the responses to Ach or SNP (one at a time) in A₂ were tested in a same manner as described in step a with the present of 10⁻⁷ M (dose 1) and 10⁻⁶ M (dose 2) L-NMMA in the suffusate (step b). Another two higher doses of L-NMMA (dose 3, 10⁻⁵ M and dose 4, 10⁻⁴ M) were applied topically while the responses to Ach or SNP were tested first in 4th order and then in 2nd order arterioles (steps c and d).



L-NMMA was added continuously into the bath during the whole procedure of the testing.

Results

Effect of varying time of light-dye treatment on acetylcholine-induced vasodilation in large (2nd Order) and small (4th Order) arterioles

To obtain the maximum loss of response to Ach in these arterioles without decreasing the vasodilator effect of SNP on the vascular smooth muscle, the time period of light-dye treatment was critical. As shown in Table 1, shorter exposure periods (<3 min) were not sufficient to abolish or significantly reduce the Ach-induced vasodilation. The exposure time for light-dye treatment in 4th order arterioles for dramatic attenuation of the Ach response was 3 minute. With this exposure time, these experiments showed that there was approximately a 95% diminution in the response to Ach in 4th order arterioles. However, the response to Ach in 2nd order arterioles was not significantly altered by prolonged light-dye treatment even to 10 min. This was evident over a wide range of acetylcholine concentrations.

Effect of endothelial impairment by light-dye treatment on vasodilator responses in different segments of the series-arranged arterioles

When either Ach or SNP were applied to 2nd order or 4th order arterioles before light-dye treatment (before L-D), there was a rapid increase in luminal diameter, which reached a maximum diameter within 10-30 sec (Fig. 10 & 11). This maximum diameter or dilation was considered the experimental diameter (D_E) and it was used to calculate the mean response for any given application as percentage change in control diameter

Table 1. Effect of varying the time of light-dye (L-D) treatment on acetylcholine-induced vasodilation in large (2nd order) and small (4th order) arterioles.

Vessel Order	Before L-D ^a			L-D Time	After L-D ^a		
	D _C	D _E	%ΔD _C		D _C	D _E	%ΔD _C
2nd order Arteriole	46	59	27	<10 min	47	57	21
	±5	±7	±3		±7	±8	±3
	(6)				(6)		
	44	52	18	10 min	44	52	20
	±2	±2	±4		±2	±2	±1
	(6)				(6)		
4th order Arteriole	19	27	36	<3 min	17	23	36
	±1	±3	±9		±1	±3	±9
	(6)				(6)		
	16	21	36	3 min	16	17	2*
	±0	±1	±2		±1	±1	±1
	(6)				(6)		

Note. Numbers in parentheses indicate number of arterioles tested. All values are means with standard errors.

a D_C, Control Vessel Diameter (μm); D_E, Experimental Diameter (μm) after Acetylcholine; %ΔD_C, Percentage Change in Control Diameter $[(D_E - D_C)/D_C](100\%)$ before and after Light-Dye (L-D) treatment. Acetylcholine concentration tested was 10⁻⁶ M in 2nd order arterioles and 10⁻⁷ M in 4th order arterioles.

* P < 0.01 by student t (paired) test, compared to data obtained before light-dye treatment.

Figure 10. Time course of acetylcholine (Ach, 10^{-7} M) and sodium nitroprusside (SNP, 10^{-7} M) responses before and after light-dye (L-D) treatment in 2nd order arterioles from one typical experiment. Ach and SNP produced a similar vasodilation in the control state (Before L-D). Light-dye treatment (After L-D) did not appear to alter the Ach response in 2nd order arteriole (top panel). Likewise, the SNP-induced vasodilation did not appear to be affected by L-D treatment either (bottom panel). Shaded bars on the time scale indicate the time periods when agonists were topically applied.

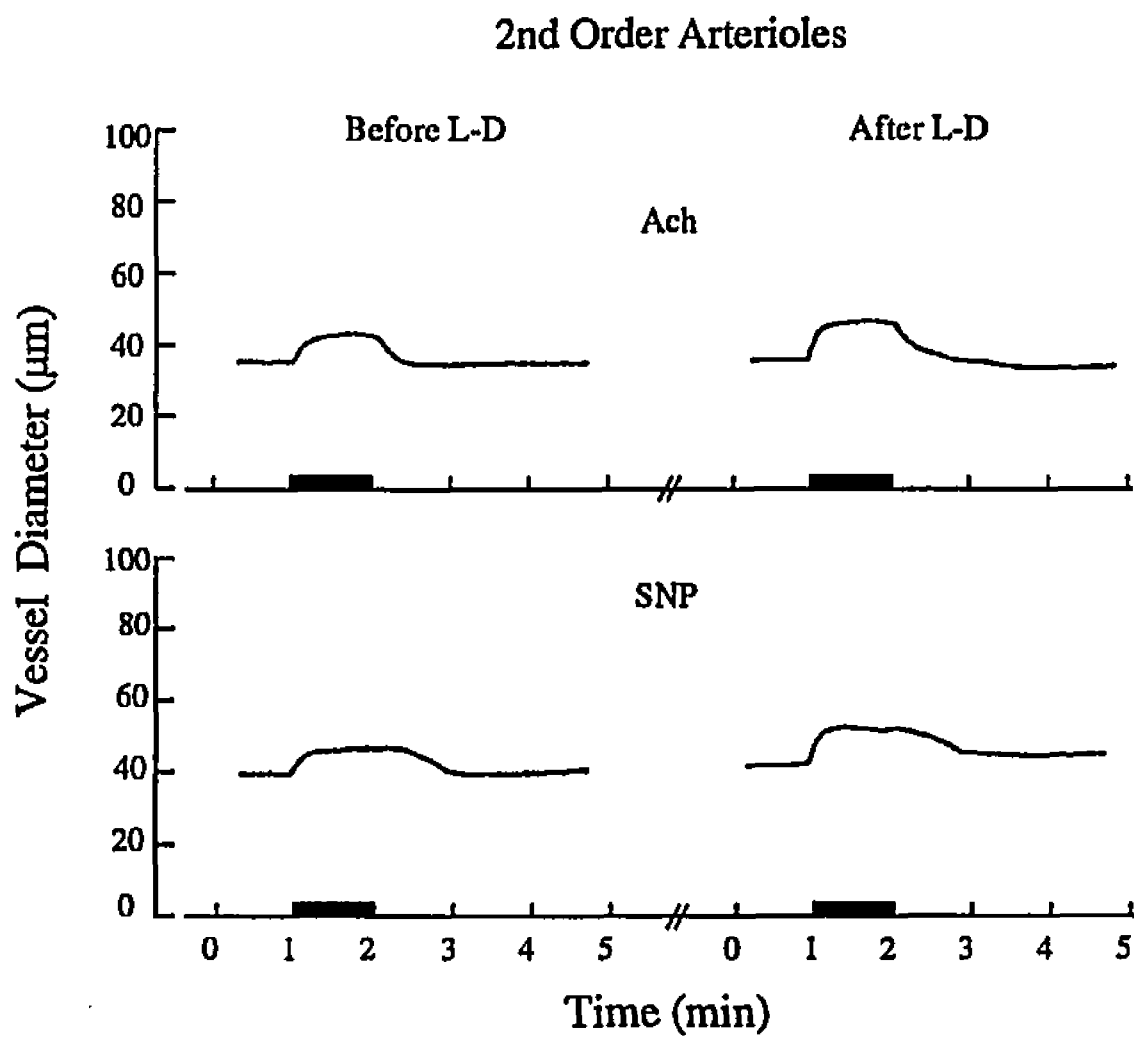
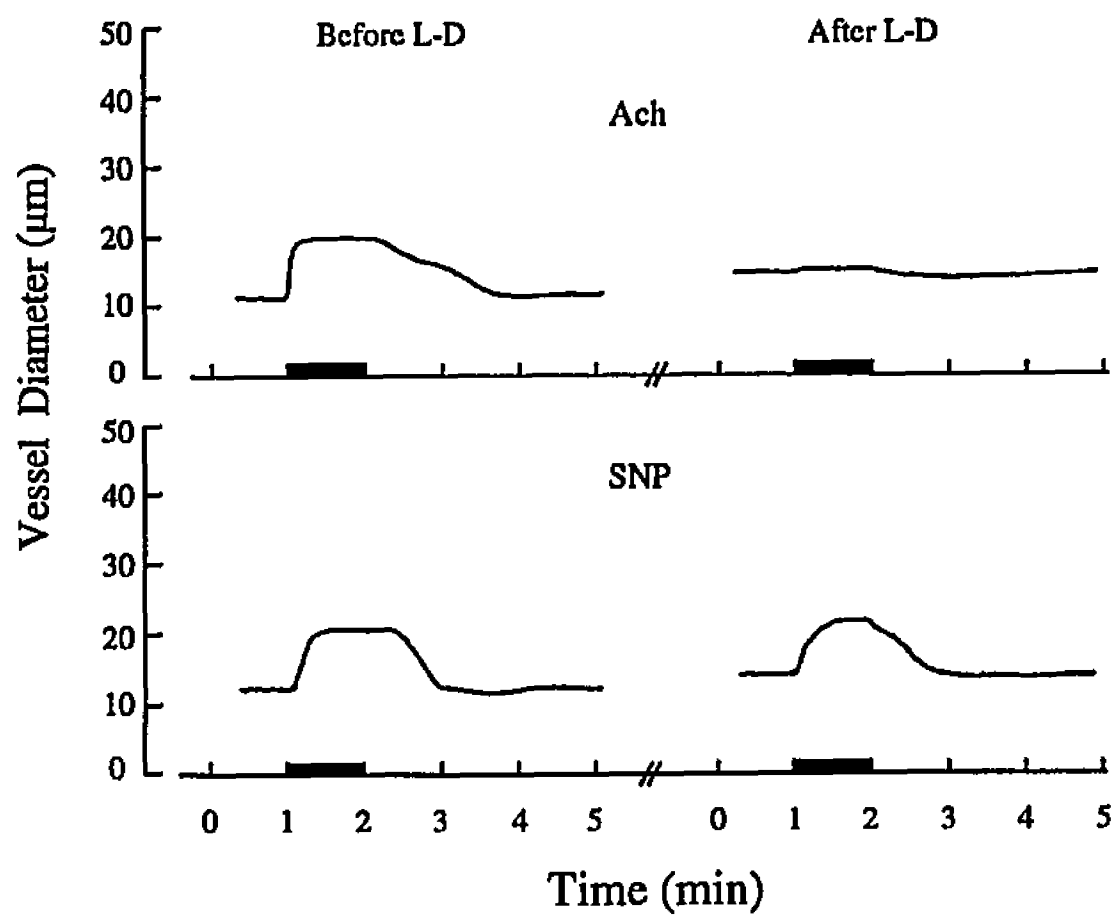


Figure 11. Time courses of acetylcholine (Ach, 10^{-7} M) and sodium nitroprusside (SNP, 10^{-7} M) responses before and after light-dye (L-D) treatment in 4th order arterioles from same experiment. Ach and SNP produced a similar vasodilation in the control state (Before L-D). Light-dye treatment (After L-D) diminished Ach-induced vasodilation in this 4th order arteriole (top panel); whereas, it did not appear to alter the SNP-induced vasodilation (bottom panel). Shaded bars on the time scale indicate the time periods when agonists were topically applied.

4th Order Arterioles



($\% \Delta D_o$). In general, the magnitude of the dilator response appeared greater in 4th order than in 2nd order arterioles. Further, after light-dye treatment (after L-D), the dilator response in 4th order arterioles to Ach (10^{-7} M) appeared to be abolished (Fig. 11, top panel), whereas it did not appear to be affected by this light-dye treatment in 2nd order arterioles (Fig. 10, top panel). Also, the dilator response to SNP (10^{-7} M) in either of these arterioles did not seem to be affected by light-dye treatment (Fig. 10 & 11, bottom panels). The effect of light-dye treatment on the control diameter of 2nd and 4th order arterioles was minimal. Statistically, there was no difference in these control diameters before versus after light-dye treatment (42 ± 3 and 16 ± 1 μ m, respectively).

Figure 12 represented the dose responses of both 2nd (top panels) and 4th order arterioles (bottom panels) to Ach and SNP before (solid lines) and after (dotted lines) light-dye treatment. In the control state, both 2nd and 4th order arterioles exhibited a dose-dependent vasodilation in response to either Ach or SNP and there was a greater responsiveness in the smaller arterioles compared to the larger arterioles for each agent (Fig. 12, solid lines). After light-dye treatment, however, the dilator responses of 2nd order arterioles to Ach and SNP were not altered (Fig. 12, top panels), whereas the dilator response of 4th order arterioles to Ach but not SNP was diminished dramatically ($P < 0.01$ in all doses applied) (Fig. 12, bottom panels).

Effect of L-NMMA on Ach-induced vasodilatory response in large (2nd order) and small (4th order) arterioles

Responses to Ach or SNP in control conditions, prior to and after L-NMMA suffusion at various concentrations for 2nd and 4th order arterioles are shown in Table 2

Figure 12. Dose-response curves for acetylcholine (Ach) and sodium nitroprusside (SNP) in 2nd and 4th order arterioles before (solid circles and solid lines) and after (open circles and dotted lines) light-dye (L-D) treatment. All values are means with standard errors as described by the percentage change in control diameter ($\% \Delta D_c$). The responses before light-dye treatment are compared to those after light-dye treatment at all doses applied (* $P < 0.05$ by student "t" (paired) test) for 2nd and 4th order arterioles. Vehicle (saline) response values are shown (■).

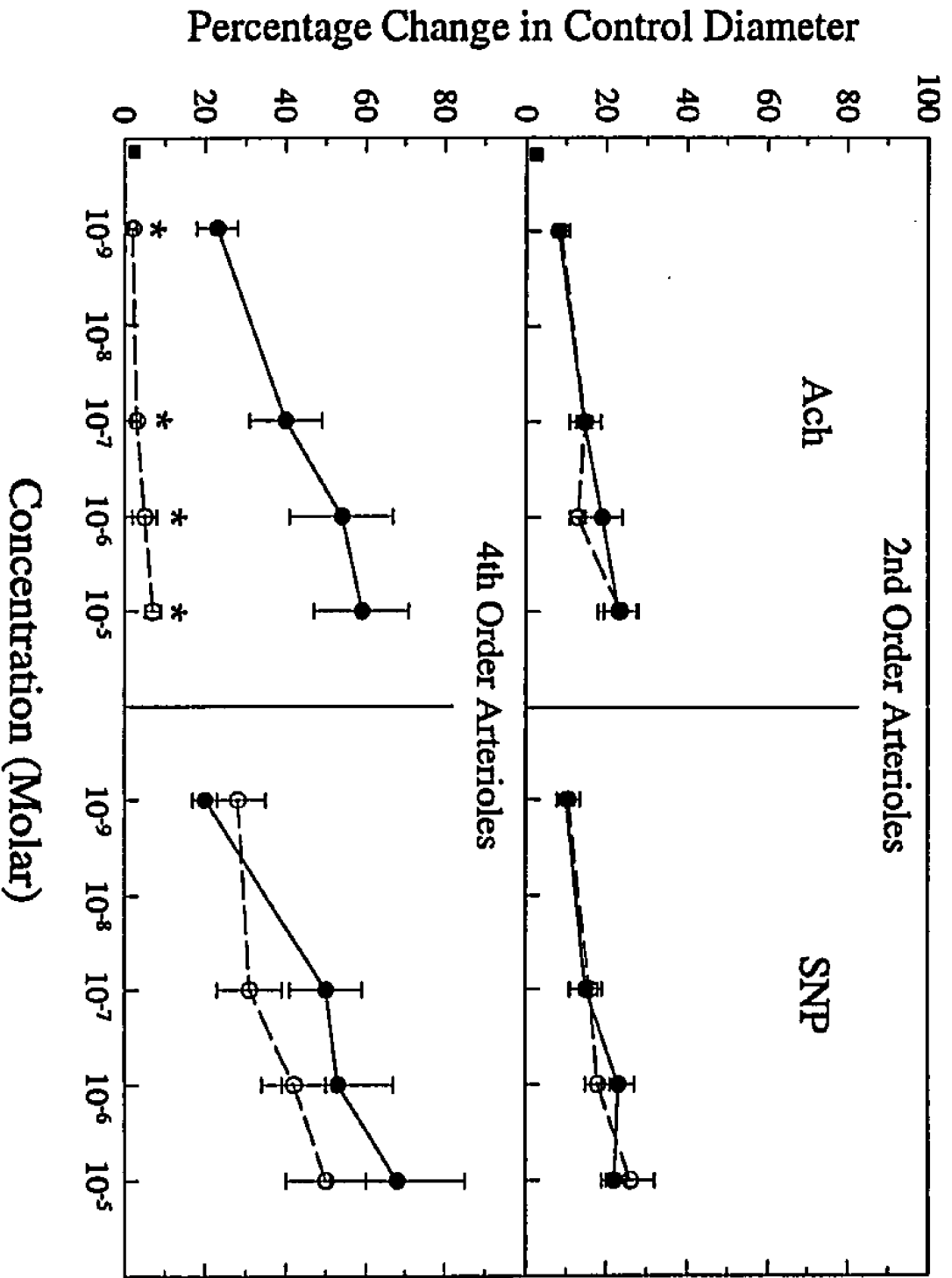


Table 2. Effect of N^G-Monomethyl L-Arginine (L-NMMA) on the responses to acetylcholine (Ach) and sodium nitroprusside (SNP) in series-arranged arterioles of the hamster cheek pouch

Vessel	Agent	D_c^a	Microvascular Responses (% ΔD_c) ^b at Various Concentrations ^c of L-NMMA				
			0	10^{-7}	10^{-6}	10^{-5}	10^{-4}
2nd Order	Ach ^d	33	36	41	43	42	55
		± 2 (8)	± 4 (8)	± 6 (3)	± 5 (8)	± 4 (8)	± 15 (5)
	SNP ^e		36	46	42	39	54
			± 2 (8)	± 7 (3)	± 4 (8)	± 5 (8)	± 17 (5)
4th Order	Ach	13	56			28*	19*
		± 1 (6)	± 9 (6)			± 4 (6)	± 6 (3)
	SNP		59			71	57
			± 7 (6)			± 19 (6)	± 11 (3)

Note. All values are means with standard errors. Numbers in parentheses represent number of hamster.

a D_c : Control Diameter (μ m).

b % ΔD_c : Percentage Change in Control Diameter ($[(D_E - D_c)/D_c](100\%)$).

c The concentration is moles/liter.

d Acetylcholine (10^{-7} M).

e Sodium Nitroprusside (10^{-7} M).

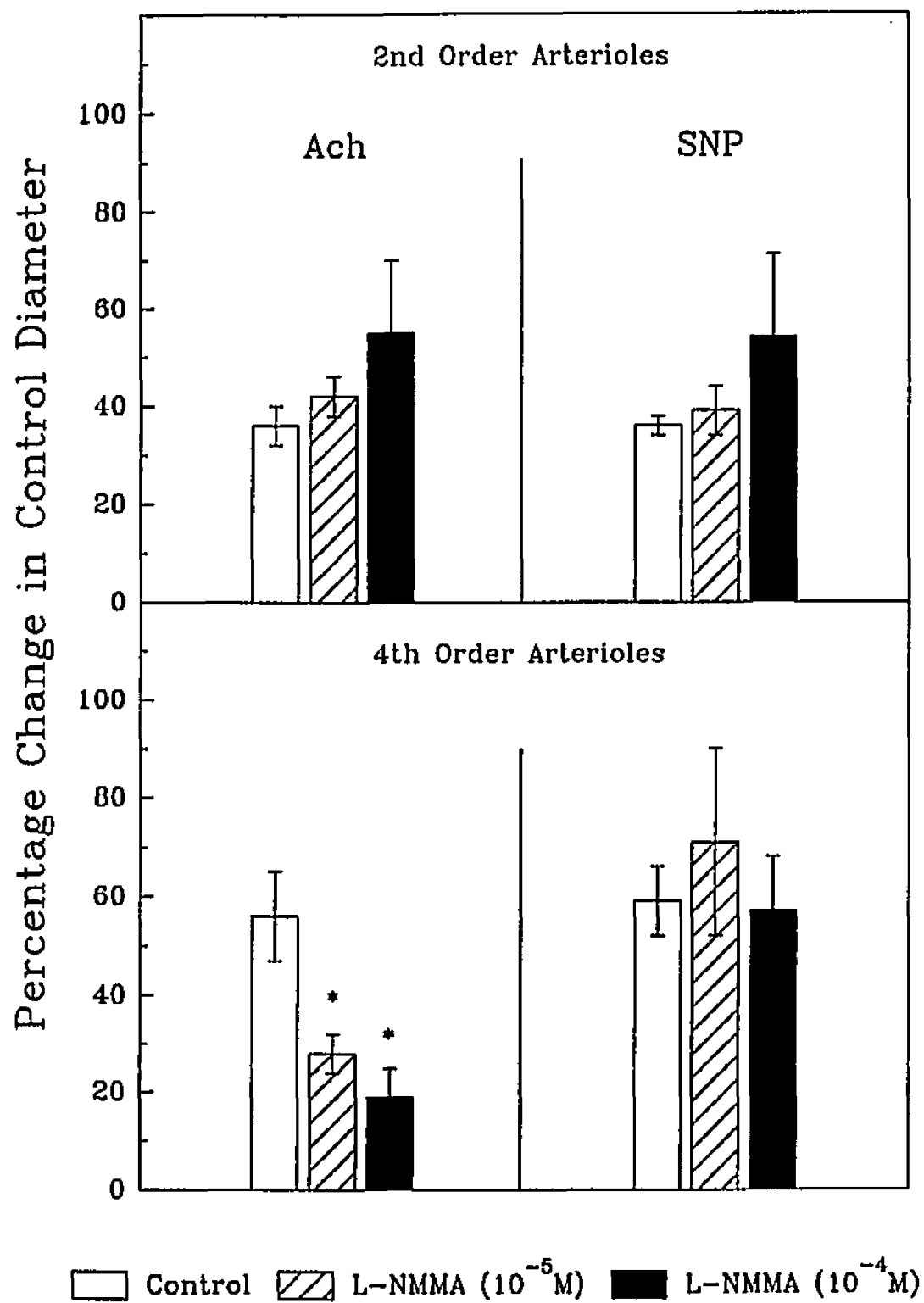
* $P < 0.01$ by student t (paired) test, compared to control (at 0 concentration of L-NMMA).

and these responses for the higher two concentrations of L-NMMA are depicted in Figure 13. In the control state, both Ach and SNP (10^{-7} M) produce a profound vasodilation in both 2nd and 4th order arterioles. After the addition of L-NMMA, the responses of 2nd order arterioles to either Ach or SNP were not altered even at the higher concentrations (10^{-5} - 10^{-4} M). In 4th order arterioles, responses to Ach were reduced significantly ($P<0.05$) in a dose-dependent manner for L-NMMA, but responses to SNP were not affected (Table 2, Fig. 13). From these data we can calculate that the response to Ach in 4th order arterioles was reduced by 50 and 66% at L-NMMA concentrations of 10^{-5} and 10^{-4} M, respectively. Further, L-NMMA (10^{-5} and 10^{-4} M) significantly suppressed Ach response in 4th order arterioles (Fig. 13, bottom panel) but did not alter Ach response in 2nd order arteriole (Fig. 13, top panel). The responses to SNP were not changed by L-NMMA in either order of arterioles. Moreover, L-NMMA treatment had no significant effect on control diameters in either 4th order ($13\pm 1\mu\text{m}$) or 2nd order ($33\pm 2\mu\text{m}$) arterioles.

Discussion and Conclusions

Many *in vitro* studies have documented that a large number of agents (e.g., acetylcholine, ATP and ADP, substance P, bradykinin, histamine, thrombin, serotonin) produce an endothelial-dependent relaxation in the conduit vessels. However, this study is the first to demonstrate heterogeneity of the Ach-induced dilation in small arterioles using both a functional impairment of the endothelium (L-D treatment) and a pharmacological blockade of EDRF formation (L-NMMA). To date, very few studies have reported endothelium-dependent relaxation *in vivo*, especially in the microcirculation. Kontos and Wei (1985) reported that in cat pial arterioles, acetylcholine

Figure 13. Effect of N^G-monomethyl-L-arginine (L-NMMA) on acetylcholine (Ach, 10⁻⁷ M, left)- and sodium nitroprusside (SNP, 10⁻⁷ M, right)-induced vasodilation in 2nd order (top panel) and 4th order (bottom panel) arterioles. L-NMMA was added continuously to the suffusion solution. All values are means with SE as described by the percentage change in control diameter (%ΔD_C). *P<0.05 by student "t" (paired) test, compare to the control.



and bradykinin induced an endothelium-dependent vasodilation. Rosenblum (1986) reported that the mouse pial arterioles demonstrated an EDRF activity which mediates the vasodilation induced by acetylcholine and bradykinin. These studies first documented the existence of EDRF in microvessels *in vivo*. Later, Phol *et al.*, (1987) and Förstermann *et al.* (1987) studied the resistance vessels of rabbit hindlimb and concluded that endothelium-mediated reactions can contribute significantly to the control of organ blood flow at the level of resistance-sized vessels (Phol *et al.*, 1987) and that this may be important for the regulation of local flow and peripheral resistance *in vivo*. Meanwhile, Furchgott *et al.* (1987) demonstrated that the major component of vasodilation for mesenteric resistance vessels in rabbit and rat by acetylcholine is mediated by EDRF. Recently, Koller and Kaley (1990) illustrated that in 50-100 μm segments of arterioles in the rat cremaster, there is an endothelium-mediated, flow-induced vasodilation, which can regulate blood flow by coupling, through endothelial mediation, blood flow velocity (wall shear stress) changes to resistance changes. These studies also documented that with those endothelium-dependent vasodilating agents, relaxation may be limited to certain species and/or blood vessels; in other words, a considerable heterogeneity of response exists among vessels from different anatomic origins and species. This study is the first to demonstrate that EDRF-like activity exists in small, 4th order (10-20 μm), arteriolar segments of the hamster cheek pouch but is not present in large, 2nd order (30-50 μm) arterioles. These results are consistent with the heterogeneity of EDRF-like activity and may indicate the diversity and specificity of endothelial cell function(s).

One of the most interesting observations is that Ach-induced EDRF-like activity was absent in larger arterioles while in the same microvascular network, Ach produces an

endothelial-dependent vasodilation in the smaller, downstream arterioles. These observation is consistent with those presented by Rivers and Duling (1986), who failed to demonstrate EDRF-like activity in larger hamster cheek pouch arterioles (50-80 μ m). This implies that there may be a different reaction for EDRF-like mechanism(s) or the larger arteriolar segments are less sensitive to stimulation of the production of EDRF-like agents in the hamster cheek pouch.

Effectiveness of light-dye treatment on endothelial impairment

Ultrastructural studies show that the damage caused by light-dye treatment is initially manifest by lucencies and vacuoles in endothelial cells and progresses to denudation only if the noxious illumination persists (Povlishock et., 1983). It was thought that light-dye treatment can impair endothelial cell function either by inactivating and/or destroying the surface receptor or by generating oxygen free-radicals, which are known to degrade EDRF quickly without producing further injury to the vessel (Povlishock *et al.*, 1983; Reed and Miller, 1988; Rosenblum, 1986; Gryglewski *et al.*, 1986; Koller *et al.*, 1989). By selecting the appropriate parameters (dye concentration, light intensity, and duration of the illumination) for a given tissue preparation, the light-dye technique appears to produce a selective inhibition of the dilator response to acetylcholine. In our intravital microscopy system, with the dye concentration of 50 mg/100 g body wt FITC-dextran (150K), the optimal duration of the illumination is 3 min for maximal selective loss of response to Ach in 4th order arterioles. Since platelet aggregation will occur after approximately 6 min of continuous light-dye treatment (Rosenblum, 1986) and longer periods of light-dye treatment can produce irreversible injury to preparations (Koller *et al.*, 1989; Reed and Miller, 1988), we tested light-dye effect on large arterioles (2nd

order) beginning at 3 min and gradually moved to longer times (10 min). The data showed that even these prolonged periods of exposure did not alter the vasodilation produced by Ach in these larger arterioles. Thus, this further indicated a much lower EDRF-like activity or less sensitivity to EDRF in these 2nd order arterioles.

Specific inhibition for the EDRF/NO pathway

The results using L-NMMA support further the hypothesis that EDRF-like activity is heterogeneously distributed along these series-arranged arterioles and this is not due to an artifact or inability to effect the Ach-sensitive mechanism(s) in endothelial cells, of the light-dye treatment.

Accumulating evidence suggests that L-arginine is the precursor of EDRF/NO and several arginine analogues are potent inhibitors of nitric oxide (NO) formation, and L-NMMA is one of these analogues. Johns *et al.* (1990) reported that L-NMMA significantly reduced bradykinin- and adenosine-triphosphate-induced cGMP formation but had no effect on nitroprusside-induced cGMP formation in bovine pulmonary endothelial and smooth muscle co-cultured cells. Similarly, the bioassay study of Ishii *et al.* (1990) reported that L-NMMA diminished A23187-induced cGMP formation in RFL-6 cultured cells in a dose-dependent manner. Rees *et al.* (1989) studied EDRF-like activity using isolated rings of rabbit aorta and found that L-NMMA inhibited Ach-induced (10^{-6} M) relaxation in a dose-dependent manner with IC_{50} of 10^{-5} M. All of these studies provide information that L-NMMA significantly inhibit EDRF-like activity at an effective concentration of 10^{-5} to 10^{-4} M during the activation with Ach. These results are consistent with our observations concerning the optimal dose of L-NMMA required to reduce a acetylcholine-induced vasodilator response.

As reviewed in Chapter 1 and illustrated in Figure 2, Ach-induced vasodilation may be generated through three possible pathways. It may activate arachidonic acid pathway in endothelial cells and in turn produce prostacyclin (PGI_2), causing vasodilation. It also may act on different subtypes of muscarinic receptors in endothelial cells and produce different factors. For example, Endothelium-Derived Hyperpolarizing Factor (EDHF) was released upon m_1 receptor activation while EDRF was released when the m_2 receptor was activated. Since both light-dye treatment and L-NMMA did not affect Ach-induced vasodilation in 2nd order arterioles, another pathway must exist for the Ach-induced vasodilation.

Consistent with the observation of Reed and Miller (1988), light-dye treatment impaired endothelial function, whereas it had no effect on the basal diameter of both 2nd and 4th order arterioles ($P>0.1$). This is also true of L-NMMA treatment. This implies a minor role of the endothelium or EDRF/NO in maintaining the basal vascular tone in the microcirculation of the hamster cheek pouch.

Conclusions

Our conclusions from these experiments are that there is a differential EDRF-like activity in arterioles of the hamster cheek pouch: activity is present in small (10-20 μm) but absent in larger (30-50 μm) arterioles. The diminished Ach response in small arterioles is due to the impairment of the endothelium but not to the damage of vascular smooth muscle cells. Other intracellular pathways that do not involve the generation of EDRF/NO from the degradation of L-arginine may be responsible for the Ach-induced vasodilation in the larger arterioles. The endothelium plays a minor role in modulating the basal vascular tone in the hamster cheek pouch microcirculation; smaller arterioles

have a higher responsiveness to vasoactive agents and this may contribute to the differential effects of endothelial impairment on the vasodilator responses.

CHAPTER 5

HETEROGENEITY OF ENDOTHELIAL CELL FUNCTION FOR ANGIOTENSIN CONVERSION IN SERIES-ARRANGED ARTERIOLES

Introduction

The vascular endothelium serves not only as a barrier to solute exchange, but also as a key modulator that plays an important role in regulating vascular tone and thus local blood flow and its distribution. It is well known that the endothelium releases various factors in response to pharmacological or mechanical stimulation. In addition, the vascular endothelium also may regulate vascular tone by modulating local angiotensin II production since it is the major site for the location of the angiotensin converting enzyme (ACE) (Kahr *et al.*, 1991; Gohlke *et al.*, 1992). Although angiotensin I (AI) is converted to a potent vasoconstrictor angiotensin II (AII) by ACE, mostly in the lung, some investigations suggest that both renin and ACE are present in the walls of the peripheral vasculature (Swales, 1979). Thus, the local generation of AII participates in the regulation of vascular tone (Oliver and Sciacca, 1984; Mizuno *et al.*, 1988; Swales and Thurston, 1973). Consequently, modulating local angiotensin conversion at different levels in the circulation may be another feature of the endothelium for the regulation of vascular tone. It has been reported also that AII stimulates the release of endothelial derived relaxing factor (EDRF) in rat aorta and bovine coronary artery and promotes the production of vasodilating prostaglandins (PG's) (Ito *et al.*, 1991; Nasjletti and Malik, 1982); these responses are endothelium-dependent. Hence, the vascular endothelium may have the potential to modulate vasoactive response to AII in the peripheral

resistance vessels (Gruetter *et al.*, 1988; Haberl *et al.*, 1990).

The regulating role of the vascular endothelium for vascular tone and blood flow distribution is complex. As reported in previous studies, there is a heterogeneity of acetylcholine(Ach)-induced EDRF activity in distinct segments of series-arranged arterioles (Tang and Joyner, 1992; Tang and Joyner, 1993). It was demonstrated that the Ach-induced dilation is mediated through an EDRF/Nitric Oxide(NO) pathway only in small (10-20 μ m) arterioles. This observation that the endothelium may play differential roles in mediating vasodilator responses in distinct segments of microvascular architectures raises new questions. For example, is the local conversion of angiotensin via an endothelial-dependent mechanism due to the localization of ACE? Further, is this endothelium-dependent mechanism homogeneously distributed throughout these series-arranged arterioles?

Although studies on isolated rat aorta, bovine coronary artery and rabbit preglomerular arteriole demonstrated the potential for a negative feedback role of the endothelium in modulating the response of AII and the local AII production (Ito *et al.*, 1991; Gruetter *et al.*, 1988), other data also indicated that the influence of the endothelium on vasoconstrictor responses to angiotensin may vary, depending upon the species and/or vascular bed (Haberl *et al.*, 1990). It is not clear for different segments of the microcirculation in the hamster cheek pouch whether alterations in the function of the endothelial cell may affect the local AII production via alterations in angiotensin conversion, or the responsiveness of the vessel to angiotensin(s).

This group of experiments were designed to test and to compare the role of the endothelium in the local conversion of angiotensin in different segments (specifically 2nd

and 4th order arterioles) of series-arranged arterioles using physical impairment of the endothelium and biochemical blockade of ACE in an *in vivo* cheek pouch preparation. The contribution(s) of the endothelium and ACE activity in the local conversion of AI to AII also is assessed. Another aim of this study is to investigate the interaction between endothelial function, i.e. the release of EDRF/NO or PG's, and the vasoconstrictor response of AII in these series-arranged cheek pouch arterioles.

Experimental Protocol

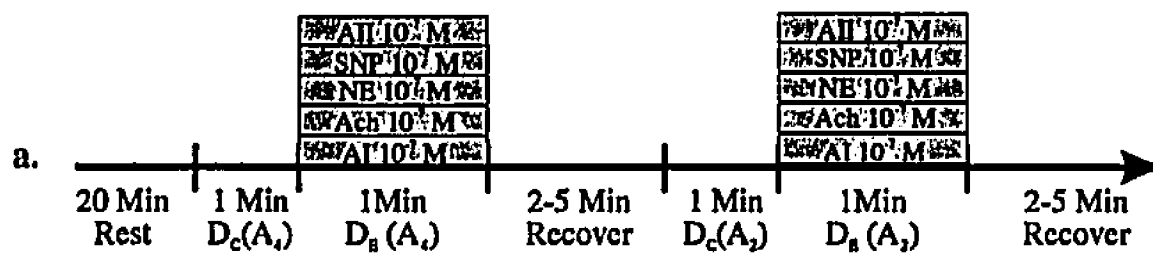
Effect of endothelial impairment using light-dye technique

The protocols for this group of experiments are illustrated in Figure 14. In selected 4th and 2nd order arterioles, control responses, as defined by changes in arteriolar diameter, were described for the constricting agents AI, AII and NE and the dilating agents Ach and SNP at test doses of 10^{-8} - 10^{-7} M. All agonists were tested in random order with the exception that tests for AI and AII were separated by tests of Ach, SNP and NE to avoid tachyphylaxis. After these control measurements, FITC-dextran was injected through the femoral vein and the previously tested arteriole was subjected to light-dye treatment. After three min (for A_1) or ten min (for A_2) of light exposure, the procedure described for control measurements was repeated on the same arteriole.

Effect of the inhibition of EDRF/NO synthesis, ACE activity or prostaglandin(s) production

Control responses to AI, AII (10^{-8} M) and Ach and NE (10^{-7} M) were tested first in 4th order and then in the upstream 2nd order arterioles, as described above. Then, the inhibitors, L-NAME, captopril (Cap) or indomethacin (Indo), were added continuously

Figure 14. Experimental protocols for testing vasoactive responses to dilators and constrictors, to determine the effect of light-dye treatment on angiotensin conversion, in large (A_2) and small (A_4) arterioles. Under the control condition (step a), responses to angiotensin I (AI), acetylcholine (Ach), norepinephrine (NE), sodium nitroprusside (SNP) and angiotensin II (AII) were tested first in A_4 and then in A_2 (one agonist at a time). Thereafter (step b), FITC-dextran was injected intravenously, and 4th order arteriole was subjected to light-dye followed by repeating step "a" in this small arteriole. Finally, 2nd order arteriole was subjected to the light-dye treatment and step "a" repeated.



into the suffusate at a rate of 0.2ml/min; the final concentrations in the suffusate were 10^{-4} , 2.3×10^{-5} and 10^{-6} M, respectively. Later, the control measurement of the response to each agonist, as stated above, was repeated while the selected blocker was present in the suffusate.

Results

Effect of endothelial cell impairment by light-dye treatment on vasoactive responses and angiotensin conversion in different segments of series-arranged arterioles

Using the light-dye treatment to impair endothelial cell function, responses of AI, AII and NE, a non-endothelial cell-mediated vasoconstrictor, were tested in large (A_2) and small (A_4) arterioles of the hamster cheek pouch. Further, to ascertain the degree of endothelial cell impairment and possible damage to the function of the vascular smooth muscle, Ach, an endothelium-dependent vasodilator, and SNP, an endothelium-independent vasodilator, were tested in the same microvascular segments. These results are described in Figures 15, 16 and 17 and summarized in Tables 3 and 4.

The time courses of AI, AII and NE-induced vasoconstrictor responses in arterioles from one cheek pouch before and after L-D treatment are shown in Figures 15 and 16. All three agents induced a rapid vasoconstriction in both large, 2nd order arterioles (Fig. 15) and small, 4th order arterioles (Fig. 16) before light dye treatment. The maximum constriction was reached within 10-30 seconds and it appeared that the time to produce the maximal constriction was slowest for AI. After L-D treatment, all agents induced constrictions but only the response to AI in small, 4th order, arterioles appeared to be diminished. The experimental diameter (D_E) was obtained when the

Figure 15. Time courses of the responses, presented as changes of arteriolar diameter (μm), to angiotensin I (AI, top panel), angiotensin II (AII, middle panel) and norepinephrine (NE, bottom panel) in 2nd order arterioles from one hamster before (left) and after (right) endothelial impairment by light-dye (L-D) treatment. The shaded bars indicate the time periods when AI and AII (10^{-8} M) and NE (10^{-7} M) were topically applied.

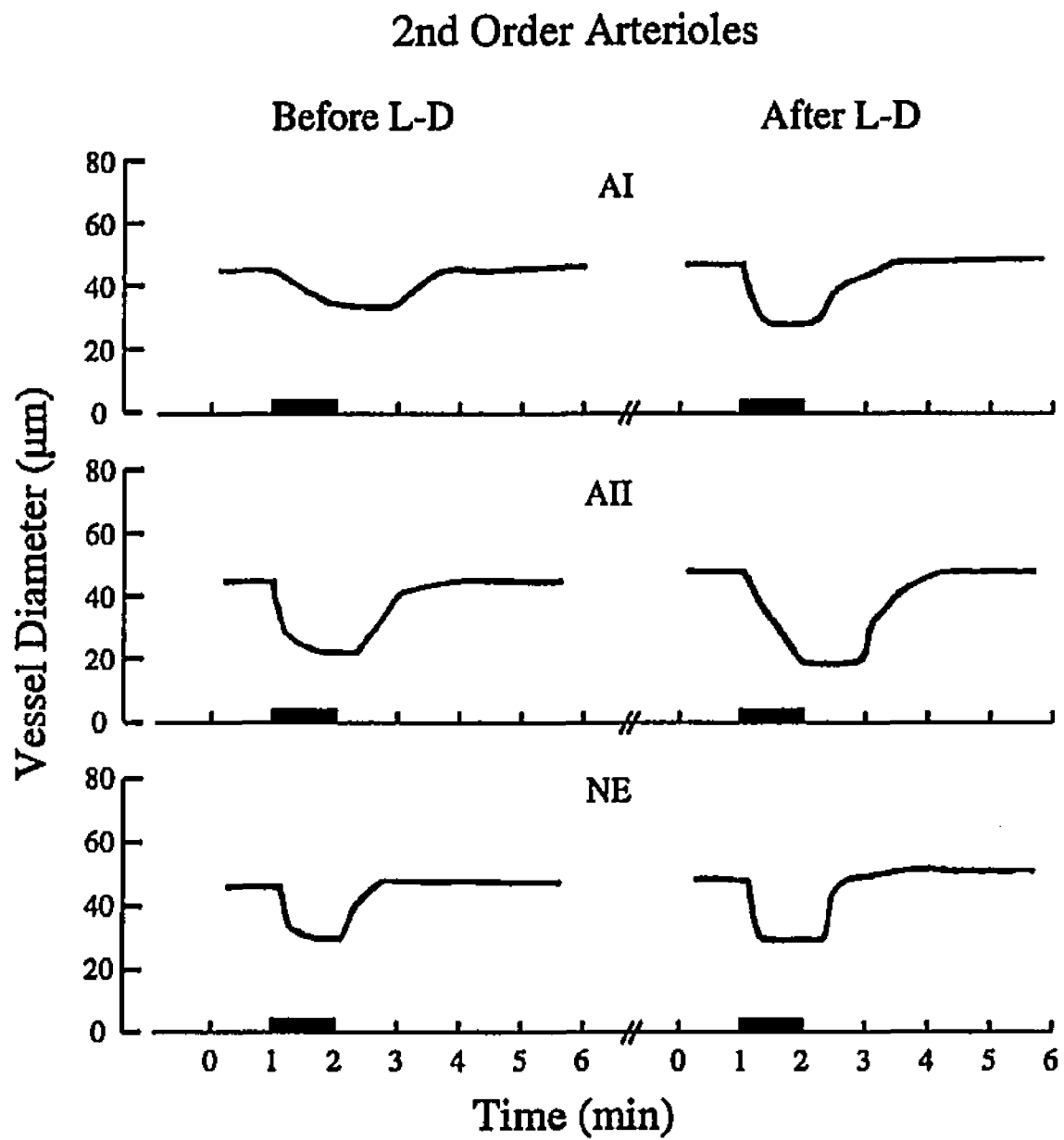
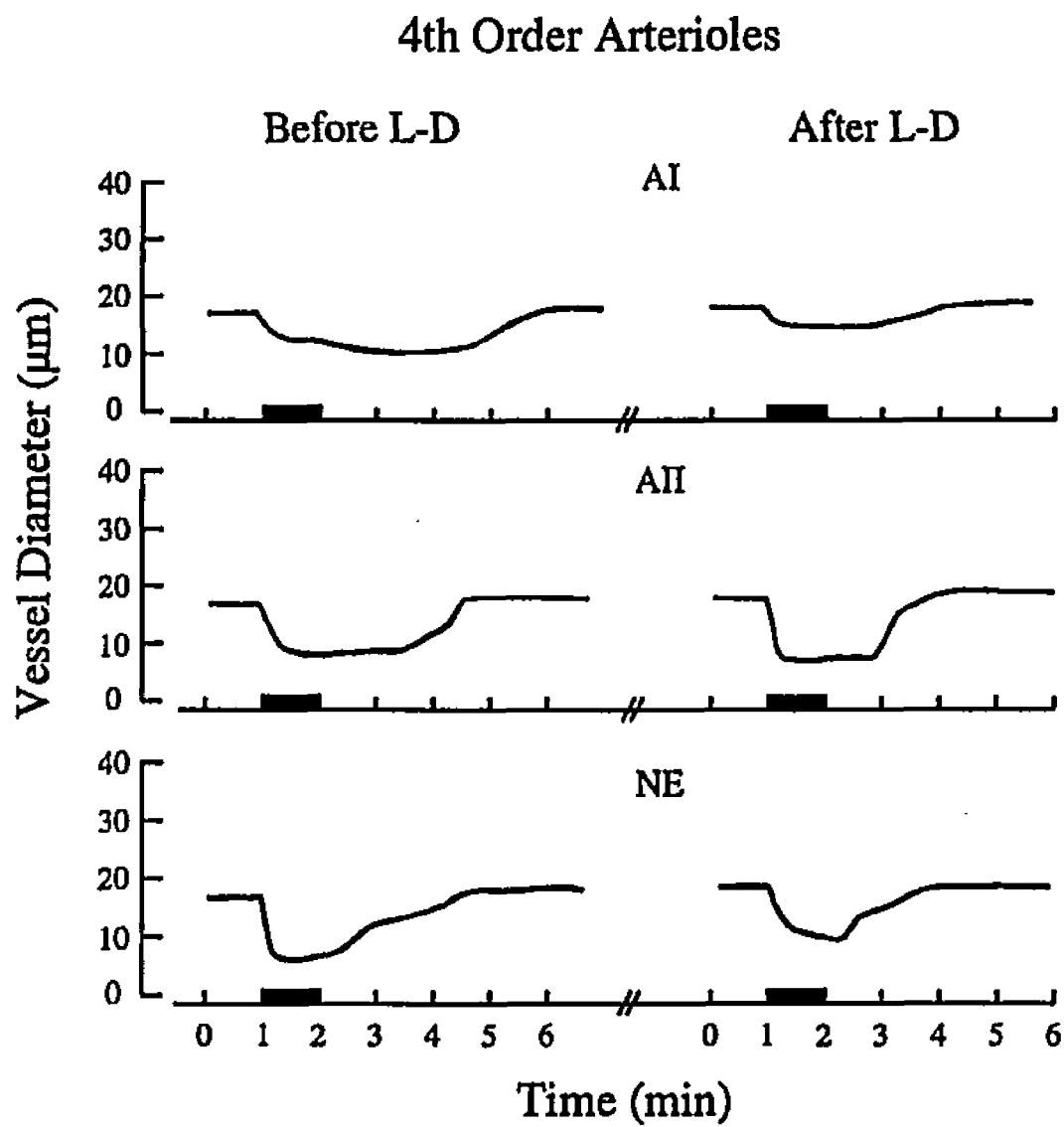


Figure 16. Time courses of the responses, presented as changes of arteriolar diameter (μm), to angiotensin I (AI, top panel), angiotensin II (AII, middle panel) and norepinephrine (NE, bottom panel) in 4th order arterioles from the same hamster before (left) and after (right) endothelial impairment by light-dye (L-D) treatment. The shaded bars indicate the time periods when AI and AII (10^{-8} M) and NE (10^{-8} M) were topically applied.

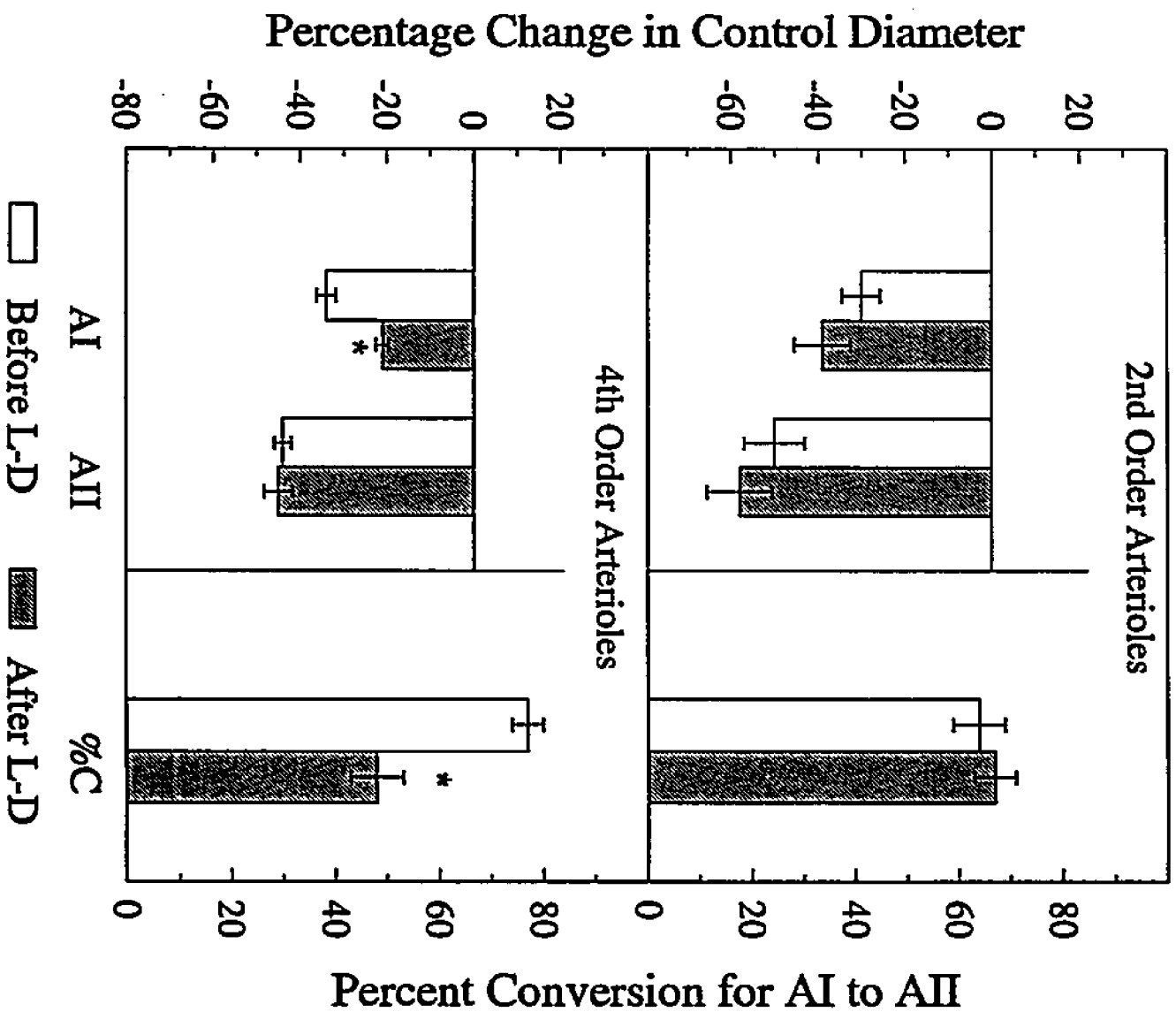


arteriole was maximally constricted and it was used to calculate the percentage change in control diameter ($\% \Delta D_c$).

As shown in Fig. 17, prior to L-D treatment, AI and AII produced a similar vasoconstriction (presented as a negative number of the percentage change in control diameter) in large (A_2) as compared to small (A_4) arterioles. Therefore, the conversion for AI to AII was not significantly different between A_2 and A_4 . After L-D treatment, in large, 2nd order, arterioles the vasoconstrictor responses to both AI and AII were not significantly changed; consequently, the calculated percent conversion for AI to AII also was not altered ($64 \pm 5\%$ vs. $67 \pm 4\%$). However, in small, 4th order, arterioles the vasoconstrictor response to AI was significantly reduced; whereas, the response to AII was not altered. Thus, the conversion for AI to AII was reduced significantly ($77 \pm 3\%$ to $48 \pm 5\%$) in these small arterioles. Using a two way analysis of variance, there was a significant interaction between L-D treatment and vessel size for percent conversion. This further illustrated the heterogenous effects of endothelial cell impairment on angiotensin conversion in these distinct arterioles (A_2 and A_4) of the hamster cheek pouch.

The vascular diameter changes induced by the application of AI, AII and NE in 2nd and 4th order arterioles before and after L-D treatment are summarized in Table 3; while, those induced by Ach and SNP are shown in Table 4. In this group of experiments, the averaged control diameters of 2nd order arterioles were 37-39 μ m; whereas, the 4th order arterioles chosen were averaged 16 μ m. It is apparent that the control diameters measured after L-D treatment were not significantly different from those obtained in the control condition for either arterioles. Furthermore, all arterioles

Figure 17. Effect of endothelial impairment by light-dye (L-D) treatment on angiotensin I (AI, 10^{-8} M, left collum), angiotensin II (AII, 10^{-8} M, middle collum) induced vasoconstriction, as described by the percentage change in control diameter ($\% \Delta D_c$), and the percent conversion for AI to AII ($\%C$, right collum) in 2nd (top panel) and 4th (bottom panel) order arterioles. Values are means with standard errors (n=6). *P<0.05 compared to controls by two way analysis of variance. Negative values indicate vasoconstriction.



recovered back to their basal diameter after the application of each agonist; hence, the basal diameters were not significantly different between the applications. As shown in Table 3, prior to L-D treatment, AI and NE produced a similar degree of vasoconstriction; therefore, the diameter of A_2 was reduced about $10\mu\text{m}$ (~30%) and the diameter of A_4 was decreased about $6\mu\text{m}$ (34-37%) by each agent. Angiotensin II however, produced a greater constriction, $19\mu\text{m}$ (50%) in A_2 and $7\mu\text{m}$ (44%) in A_4 . After L-D treatment, in 2nd order arterioles, the vasoconstrictor responses to all three agents were not significantly altered; while, in 4th order arterioles, the responses to AII and NE were not affected by L-D treatment but the constrictor response to AI was significantly diminished.

In this same series of experiments, the response to an endothelial-dependent dilator, acetylcholine, and an endothelial-independent dilator, sodium nitroprusside, also were tested to monitor the function of the endothelium. As shown in Table 4, Ach and SNP similarly dilated 2nd order arterioles of about $7\mu\text{m}$ (19%), and these responses were not reduced by L-D treatment. In 4th order arterioles, Ach and SNP produced comparable dilations of about $6\mu\text{m}$ (37%) in the control state. Light-dye treatment did not alter the dilation induced by SNP; however, it abolished the Ach-induced dilation in these small arterioles.

Effect of ACE blockade

To evaluate the contribution of ACE in conversion of angiotensin in different segments of series-arranged arterioles, a series of experiments were conducted using Cap, an inhibitor of ACE activity, to probe the interaction of the microvascular responses to AI, AII with the enzyme ACE (Figure 18 and Table 5). In the control state, AI and AII

Table 3. Effect of specific vasoconstrictor agents on large and small arterioles of the hamster cheek pouch before and after light-dye (L-D) treatment.

Agent	Control			After L-D treatment		
	D _C ^a	D _E ^b	%ΔD _C ^c	D _C	D _E	%ΔD _C
Large Arterioles - 2nd order						
AI	37 ±3	27 ±3	-30 ±4	37 ±3	22 ±3	-39 ±7
AII	39 ±3	20 ±4	-50 ±7	38 ±3	17 ±1	-58 ±7
NE	38 ±4	27 ±2	-29 ±3	36 ±4	24 ±3	-33 ±3
Small Arterioles - 4th order						
AI	16 ±1	11 ±2	-34 ±2	16 ±1	13 ±1	-21* ±2
AII	16 ±1	9 ±1	-44 ±2	16 ±1	9 ±1	-45 ±3
NE	16 ±0	10 ±1	-37 ±6	17 ±1	11 ±1	-33 ±4

a Control diameter(μm).

b Experimental diameter(μm).

c Calculated percentage change in control diameter.

* Significant different from the control (P<0.05) (by two way analysis of variance)

All values are means with standard errors, n=6.

Angiotensin I (AI) and angiotensin II (AII) were 10⁻⁸ M; whereas, norepinephrine (NE) was 10⁻⁷ M.

Table 4. Effect of specific vasodilator agents on large and small arterioles of the hamster cheek pouch before and after light-dye (L-D) treatment.

Agent	Control			After L-D treatment		
	D_c^a	D_E^b	$\% \Delta D_c^c$	D_c	D_E	$\% \Delta D_c$
Large Arterioles - 2nd order						
Ach	36 ± 3	43 ± 3	19 ± 2	37 ± 3	45 ± 3	20 ± 2
SNP	38 ± 3	45 ± 3	19 ± 2	36 ± 4	45 ± 3	27* ± 4
Small Arterioles - 4th order						
Ach	16 ± 1	21 ± 1	37 ± 3	16 ± 1	16* ± 1	1* ± 1
SNP	16 ± 1	22 ± 2	37 ± 4	16 ± 1	21 ± 1	34 ± 2

a Control diameter (μm).

b Experimental diameter (μm).

c Calculated percentage change in control diameter.

* Significant different from the control ($P < 0.05$) (by two way analysis of variance)

All values are means with standard errors, $n=6$.

Acetylcholine (Ach) and sodium nitroprusside (SNP) were 10^{-7} M.

induced similar vasoconstrictor responses to those observed for the group using light-dye treatment. After addition of Cap in the suffusate, the vasoconstrictor responses to AII were not significantly altered in either arterioles (A_2 or A_4). However, the vasoconstriction induced by AI in both 2nd and 4th order arterioles was significantly inhibited by captopril (Figure 18). In fact, in the small arterioles (A_4), after the application of Cap, AI produced a slight vasodilation instead of a vasoconstriction. Therefore, the calculated percent conversion for AI to AII was abolished completely. In 2nd order arterioles, the vasoconstrictor responses to AI but not AII were diminished and the calculated percent conversion was significantly ($P<0.05$) reduced by about 50% (Figure 18).

The vasoactive responses to NE and Ach were tested in this group also to monitor the contractility of vascular smooth muscle and the interaction of endothelial cell function with ACE. The results are summarized in Table 5. The basal diameters were $40\pm 2\ \mu\text{m}$ in A_2 and $15\pm 1\ \mu\text{m}$ in A_4 . Application of Cap did not alter these basal diameters; furthermore, ACE blockade had no significant effect on NE in either of these arterioles. Also, the response to Ach tested in this series of experiments was not significantly altered by treatment with Cap in 2nd and 4th order arterioles.

Effect of EDRF/NO and prostaglandin(s) synthesis blockade

To assess the involvement of EDRF/NO in modulating the vasoconstrictor responses to angiotensin, an inhibitor of EDRF/NO synthesis, L-NAME($10^{-4}\ \text{M}$), was applied topically. To assure the inhibition of EDRF/NO activity, the response to Ach was also tested in this series of experiments. The results are described in Table 6. The average basal diameters in this group for large and small arterioles were $35\pm 2\ \mu\text{m}$ and

Figure 18. Effect of angiotensin converting enzyme blockade by captopril ($2.3 \times 10^{-5} \text{M}$) on angiotensin I (AI, 10^{-8}M , left collum) and angiotensin II (AII, 10^{-8}M , middle collum) induced vasoconstriction responses, as described by the percentage change in control diameter ($\% \Delta D_c$), and the percent conversion for AI to AII (%C, right collum) in 2nd (top panel) and 4th (bottom panel) order arterioles. Values are means with standard errors (n=6), positive indicates vasodilation and negative indicates vasoconstriction. *P<0.05 compared to control by two way analysis of variance.

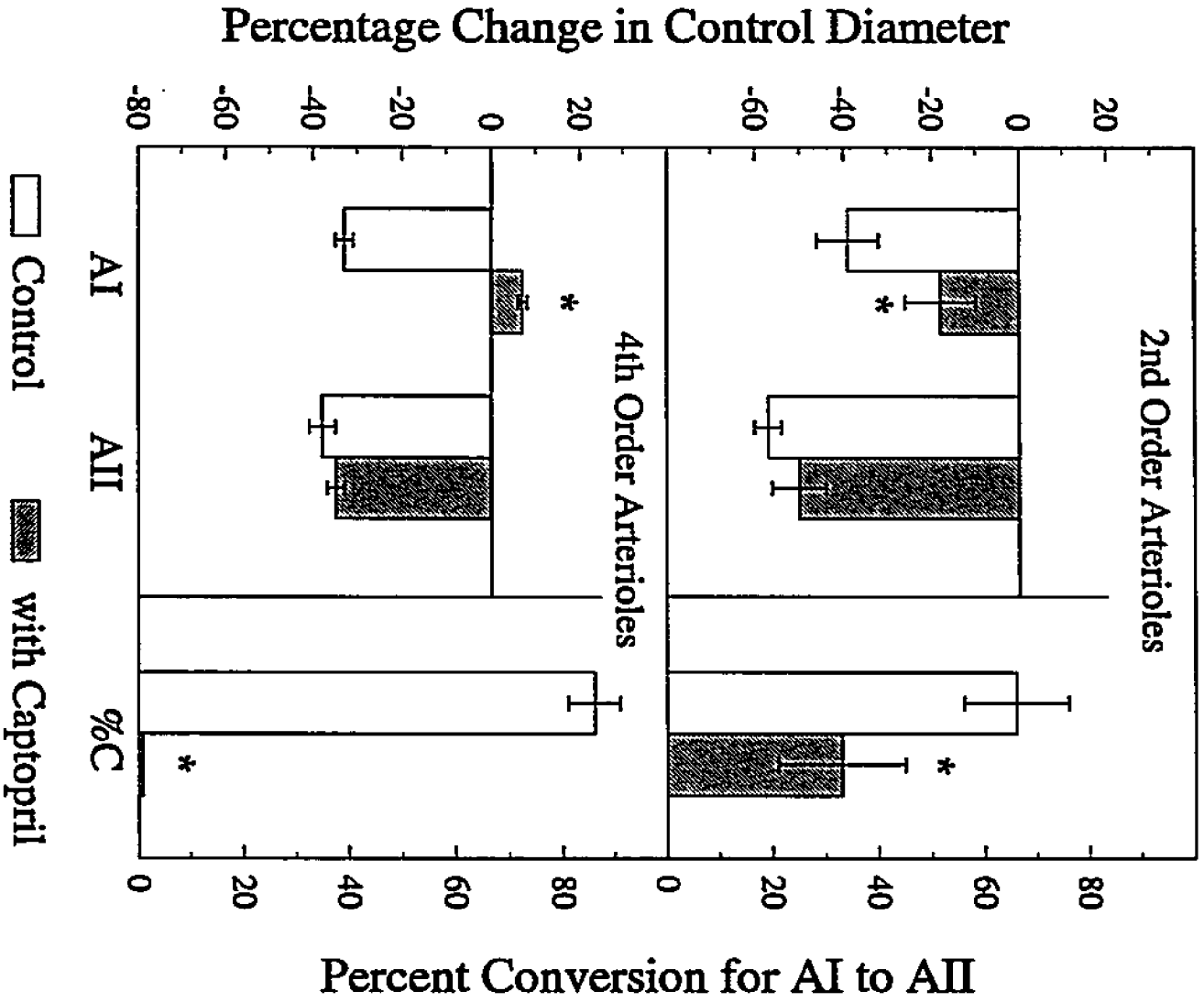


Table 5. Effect of ACE blockade using captopril (Cap) on vasoconstrictor and vasodilator responses to norepinephrine (NE) and acetylcholine (Ach)

Arteriolar Vessel Order		Vessel Response ^a	
		NE	Ach
2nd (40±2µm) ^b	Control	-19 ±2	21 ±3
	Cap	-14 ±3	18 ±4
4th (15±1µm) ^b	Control	-20 ±2	33 ±5
	Cap	-26 ±4	33 ±3

Note. All values are means with their standard errors (n=6).

a Vessel Response was expressed as percentage change in control diameter (% ΔD_c). Negative numbers indicate vasoconstriction while positive numbers indicate vasodilation.

b Averaged basal diameter in micrometers.
Ach and NE were 10^{-7} M, Cap was 2.3×10^{-5} M.

Table 6. Effect of EDRF/NO blockade using N^w-nitro-L-arginine methyl ester (L-NAME) on vasodilator responses to acetylcholine (Ach) and vasoconstrictor responses to angiotensin II (AII)

Arteriolar Vessel Order		Vessel Response ^a	
		Ach	AII
2nd (35±2µm) ^b	Control	22 ±5	-54 ±4
	L-NAME	32 ±5	-47 ±5
4th (14±0.4µm) ^b	Control	39 ±4	-35 ±3
	L-NAME	10* ±2	-30 ±3

Note. All values are means with their standard errors (n=5).

a Vessel Response: percentage change in control diameter (%ΔD_c). Negative numbers indicate vasoconstriction while positive numbers indicate vasodilation.

b Averaged basal diameter in micrometers.

* P<0.05 by Student t (paired) test compare to control.

Ach and NE were 10⁻⁷ M, L-NAME was 10⁻⁴ M.

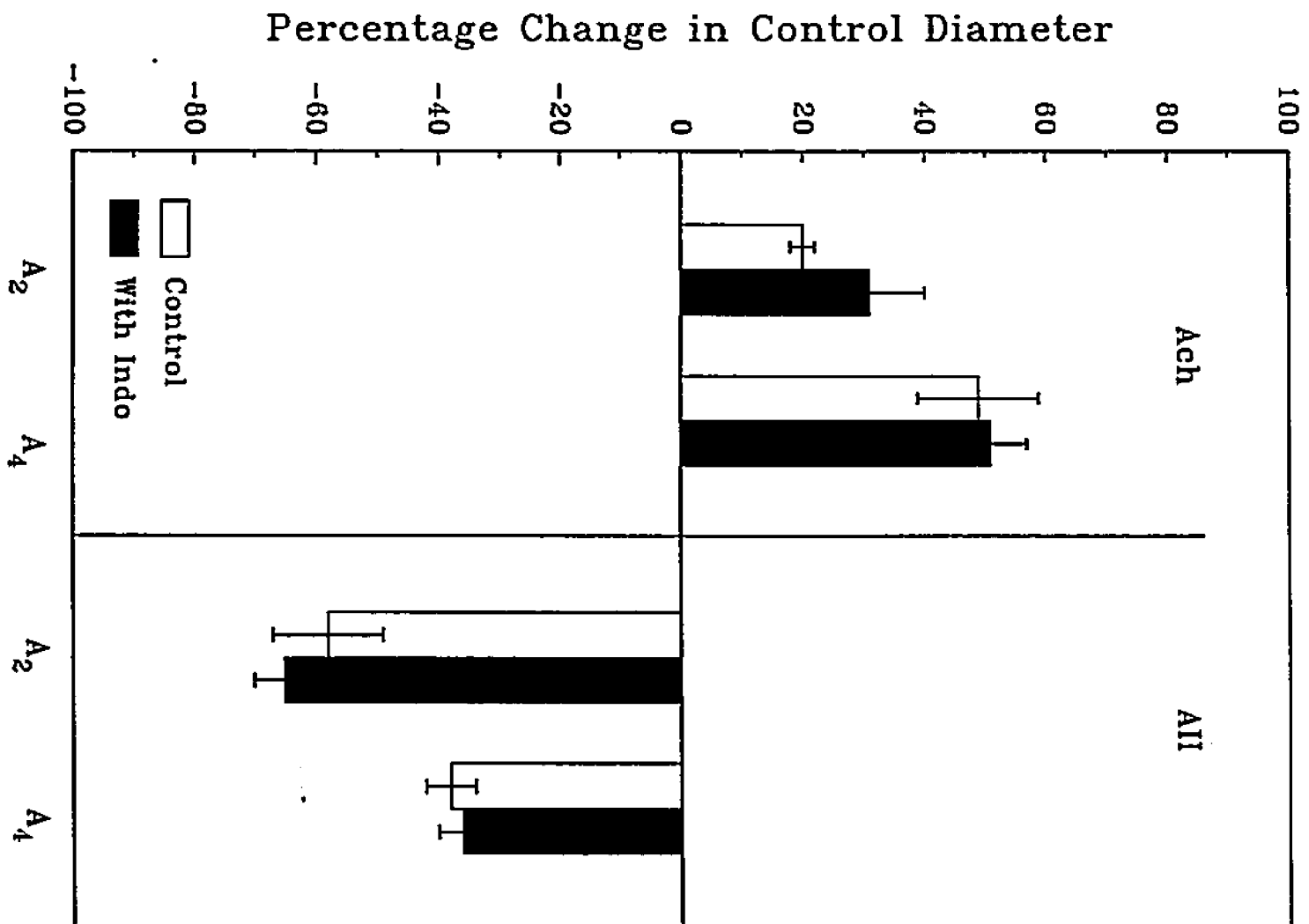
14±0.4µm, respectively and they were not significantly changed by L-NAME. In the control state, Ach produced vasodilation, 22% in A₂ and 39% in A₄ and these were comparable to those obtained in the L-D treatment group. Angiotensin II, however, produced vasoconstriction by 54% in A₂ and 35% in A₄. After the application of L-NAME, the EDRF activity was significantly inhibited, as demonstrated by an diminished Ach response (P<0.05), in 4th order arterioles; whereas, the constrictor response to AII was not altered. In 2nd order arterioles, on the other hand, the application of L-NAME had no significant effect on the responses to either Ach or AII.

The role of prostaglandin(s) in mediating Ach or AII responses in microvessels of the hamster cheek pouch was tested using a cyclooxygenase inhibitor, indomethacin. The averaged basal arteriolar diameter of this group was 43±2µm in A₂ and 14±1µm in A₄. The results are plotted in figure 19 (Mean±SE, n=4). In the control state, acetylcholine produced 20% and 31% dilations in A₂ and A₄, respectively. Angiotensin II, on the other hand, induced potent constrictions in both 2nd (58%) and 4th (38%) order arterioles. The application of indomethacin did not statistically alter responses to AII and Ach in either order of arterioles.

Discussion and Conclusions

This study demonstrates the heterogeneity of endothelial function in modulating angiotensin conversion in different segments of these series-arranged arterioles. These data show that in small (4th order) but not in large (2nd order) arterioles, the impairment of endothelial cell function by light-dye (L-D) treatment significantly attenuates angiotensin conversion for AI to AII; therefore, the local angiotensin production in 4th order arterioles is dependent, to some degree, on the function of the vascular endothelial cells.

Figure 19. Effect of indomethacin (Indo, 10^{-5} M) on vasoactive responses to angiotensin II (AII, 10^{-8} M, right) and acetylcholine (Ach, 10^{-7} M, left), as described by percentage change in control diameter ($\% \Delta D_c$). Values are means ($n=4$) with standard errors, positive indicates vasodilation and negative indicates vasoconstriction. No statistical differences were found between data obtained under control and Indo treated conditions.



Effectiveness of light-dye treatment

Several studies have reported that L-D treatment is a practical method for impairing endothelial cells without damaging the vascular smooth muscle cells. Rosenblum (1986) using mouse pial arterioles, Koller *et al.* (1989) using rat cremaster arterioles and in this lab (Tang and Joyner, 1992) using hamster cheek pouch arterioles have all demonstrated that Ach-induced or shear stress-induced vasodilation was inhibited significantly by endothelial impairment using L-D treatment. The damaging effect of L-D treatment on endothelial cell depends on the dose of fluorescein (FITC), the intensity of the illuminating light and the duration of the exposure to the excitation wavelength of light. In our system, at a FITC dose of 50 mg/100 g b.w. (Svensjö and Joyner, 1983) we demonstrated a significantly diminished Ach response in 4th order arterioles by three minutes of L-D treatment. However, prolongation L-D treatment up to 10 minute had no effect on the Ach response in 2nd order arterioles. According to Rosenblum *et al.* (1987), the ultrastructure of the endothelial cells showed morphological changes, e.g. some degree of lucencies, vacuoles and blebs after 5-6 minutes of L-D treatment. Koller *et al.* (1989) reported selectively impaired of the Ach response in 50-100µm cremaster arterioles after 2-3 minutes of L-D treatment. In addition, Haberl *et al.* (1990) demonstrated that with 1.5 minutes of L-D illumination, both the Ach- and the AII-induced dilation was reversed to constriction in pial arterioles (11-73 µm) of the rat. All of these studies illustrated very toxic effects of L-D treatment on endothelial cells. Therefore, our data showing a diminished Ach response in small, 4th order, arterioles and an unaltered Ach response in large, 2nd order, arterioles after prolonged L-D treatment suggest that the vasoactivity dependent upon functional endothelial cells is not

homogeneously distributed in all segments of these series-arranged arterioles in the hamster cheek pouch. The present study provides further evidence indicating this heterogeneity of endothelial-dependent vasoactivity in different segments of the microvessels in response to angiotensin(s) since the conversion of AI to AII in small (4th order) but not in large (2nd order) arterioles is sensitive to L-D treatment. It is likely that in these larger arterioles, the regulatory mechanisms for control of blood flow are different from those in smaller arterioles. This could be due to various factors, e.g. different receptor or enzyme distributions, specific signal transduction pathways or the distinct structures of the vascular walls.

Vascular endothelial cell may not be the only location for angiotensin conversion enzyme

In 4th order arterioles, angiotensin conversion is completely abolished by blocking the angiotensin converting enzyme; whereas, the conversion is partially reduced (38%) by endothelial impairment using the L-D treatment. This indicates that, in these small arterioles, ACE is the only pathway for the conversion for AI to AII. Since the L-D treatment only partially reduced angiotensin conversion, two possibilities may be involved: 1) the L-D treatment itself may only fractionally impair the activity of ACE located within the vascular endothelial cells, and 2) ACE inside the vascular wall may not all be located in the endothelial cells. It seems that at least part of the reduced angiotensin conversion by L-D treatment is due to the impaired ACE activity. On the other hand, in 2nd order arterioles, endothelial impairment does not affect angiotensin conversion, while captopril inhibits this conversion by 50%. This suggests that the endothelial cells may not be the major or the only pathway for the conversion of angiotensin; some other enzymes maybe involved in angiotensin conversion in these

larger arterioles.

Okunishi *et al.* (1984) studied angiotensin conversion in blood vessels isolated from dogs and monkeys and they demonstrated that another enzyme or pathway other than ACE converts AI to AII was present in vascular tissues and lungs. It may play an important role in the local generation of AII, which possibly regulates the regional vascular tone. Cornish *et al.* (1979) studied angiotensin conversion in the hamster cheek pouch and they found that after blockade of ACE, there was still 68% of AI converted to AII. Thus, they concluded that the vasculature of the hamster cheek pouch converts significant amounts of AI to AII by a route that does not involve ACE. Juul *et al.* (1987) using an angiotensin conversion assay found that functional removal of endothelial cells in isolated rat resistance vessels (200 μ m) had little effect on the contractile response to either AI or AII. However, Pipili *et al.* (1989) reported that ACE activity was reduced by approximately 30% after removal of the endothelium by chemical or mechanical methods. All of these studies were completed using microvessels or arterioles larger than 200 μ m and were consistent with our data showing that in large, 2nd order arterioles, the conversion of angiotensin is not altered by endothelial impairment; whereas, it was only partially inhibited by ACE blockade. These results are consistent with the hypothesis that the endothelial cell may play a relatively minor role for angiotensin conversion in these large arterioles or that some other counteracting pathways are involved.

Function of the smooth muscle cells after light-dye treatment

The results showing the failure of angiotensin I to induce a vasoconstriction in A₄ after L-D treatment could be attributed to dysfunctional smooth muscle cell(s). However, since the response to a non-endothelial mediated vasoconstrictor,

norepinephrine (or to AII), was not altered by L-D treatment in either large or small arterioles, the reduced AI response in small, 4th order, arterioles after L-D treatment does not appear to be due to the failure of the smooth muscle cell to contract. Instead, it is more likely that angiotensin conversion in small arterioles is dependent, to some degree, on the function of the endothelial cells. This conclusion is supported further by the response of a non-endothelial mediated dilator, sodium nitroprusside(SNP). As shown in Table II, the response to SNP in either arterioles was not reduced, indicating an unchanged dilatory feature of the vascular smooth muscle cells after L-D treatment. In fact, the SNP response in 2nd order arterioles was enhanced ($P<0.05$ by Tukey test) after endothelial impairment using L-D treatment. This could be associated with the specific supersensitivity of the nitrovasodilators described by Moncada *et al.* (1991) in that the removal of endothelial cells, or the inhibition of nitric oxide, increased the sensitivity to nitrovasodilators.

Considerations about tachyphylaxis, neural control and other circulating agents

It has been reported that the vasoconstrictor response of cheek pouch arterioles to angiotensin II showed a reduced responsiveness over time, i.e. , tachyphylaxis is present in the cheek pouch preparation (Mohama, *et al.*, 1984). To avoid tachyphylaxis, the repeated administration of angiotensin(s) was completed at a site 50-100 μm away from the site previously tested. In most of our experiments, AI was tested prior to the test of AII; while, the vasoconstrictor response to AII in either arterioles was not altered by L-D treatment. Therefore, the decreased response to AI in small arterioles after L-D treatment was not the effect of tachyphylaxis. This conclusion also is supported by the study of Vicaut *et al.* (1989) showing that distal microvessels had less tachyphylaxis

compared to proximal vessels, although the study was conducted in rat cremaster arterioles.

In some experiments, systemic blood pressure was monitored via the femoral artery and it was not affected by topical application of either agonists or antagonists. Together with the fact that the microvessels in the hamster cheek pouch appear to lack adrenergic innervation (Joyner *et al.*, 1983), it is reasonable to assume that external neural or adrenergic factors do not contribute significantly to the local vasoactive responses described in this study. Our results would be consistent with modulation of vascular cell function by local, microvascular pathways.

Since another component of ACE activity is the degradation of bradykinin and since bradykinin is a potent vasodilator, the effective potentiation of this vasodilation by inhibiting ACE activity with Cap is a concern. In this study, although the concentration of Cap used is high enough to potentiate the activity of bradykinin (Rhaleb *et al.*, 1989), the basal diameters were not changed after the application of Cap. This implies that circulating levels of bradykinin or local degradation do not have significant effects on the basal condition of these vessels; also, bradykinin (or its degradation) was not responsible for the reduced response to AI since the response to AII was not altered after Cap application.

Role of endothelial cells and the involvement of EDRF/NO or prostaglandins in angiotensin vasoactive response.

As mentioned earlier, there are some studies reporting that the endothelium modulates AII responses by releasing either EDRF or PG's. Gimbrone (1975) reported that AII stimulates PG's production in cultured human endothelial cells. Later, Nasjletti

and Malik (1982) pointed out that AII induces release of PG's from a variety of organs including the kidney, heart and blood vessels. The generated PG's (most likely PGE₂ and PGI₂) counteract the vasoconstrictor action of AII and this may be a feature of negative feedback, i.e. processes modulating their own vascular actions. In addition to the release of PG's, some groups have shown the release of EDRF by AII. For example, Eglème *et al.* (1990) reported that the endothelium modulates the responses to either exogenous and endogenous AII through the release of EDRF. Furthermore, Haberl *et al.* (1990) reported that instead of vasoconstriction, AII (10^{-10} M- 10^{-5} M) induced an endothelial-dependent dilation in rat pial arterioles. This response was reversed to a constrictor response by: the removal of the endothelium, the inhibition of EDRF/NO release using methylene blue and the inhibition of cyclooxygenase using indomethacin. Our data is not consistent with these observations, in that both endothelial impairment using L-D treatment and an inhibitor of EDRF/NO synthesis/release, L-NAME, did not alter the AII vasoconstrictor responses. Since the dose of L-NAME did not alter the Ach response in large, 2nd order, arterioles, implying failure of this inhibition to reduce the synthesis/release of EDRF/NO, two higher doses of L-NAME (3×10^{-4} M, 10^{-3} M) were tested and they did not reduce the Ach response in these large arterioles (data not show). This provides further evidence supporting the lack of EDRF/NO activity in these larger arterioles. Likewise, an inhibitor of PG's synthesis, indomethacin, also had no effect on the AII response in these arterioles (Figure 19). These results could be due to the lack of the AII receptors on the endothelial cell in these cheek pouch microvessels or the different intracellular signaling pathways among the different species and vessels.

Lastly, it appears that Ach-induced dilation is not mediated by prostaglandins since

indomethacin did not alter the Ach vasodilator response in these cheek pouch arterioles. The mystery concerning the pathway for mediation of the Ach-induced dilation in large, 2nd order arterioles needs further investigation.

Conclusions

In summary, endothelial cells play differential roles in modulating angiotensin conversion in distinct segments of series-arranged arterioles in the hamster cheek pouch. Although the basal release of EDRF did not affect the vasoconstrictor responses to AI, and/or AII, a functional endothelium is crucial for angiotensin conversion in small, 4th order, arterioles (10-20 μm). Moreover, part of the conversion appears to be independent of ACE in large, 2nd order, arterioles. Therefore, the regulatory and/or transduction mechanism(s) for the control of arteriolar diameter and subsequently, the resistance and the distribution of blood flow may be different in these distinct segments of series-arranged arterioles. Finally, it is likely that AII vasoconstrictor responses in these cheek pouch arterioles are not modulated by endothelial cells through the pathway involving the release of EDRF/NO or prostaglandin(s).

CHAPTER 6

MICROVASCULAR AUTOREGULATION: DIFFERENTIAL FEATURES
OF SERIES-ARRANGED ARTERIOLES IN THE HAMSTER CHEEK
POUCH

Introduction

The myogenic response has been defined as the property of blood vessels to maintain a constant blood flow over a certain range of arterial pressure by altering their internal diameter and /or velocity of flow. The myogenic response has been demonstrated in several vascular beds, including hamster cheek pouch, by both *in vivo* and *in vitro* studies (Gilmore *et al.*, 1980; Jackson and Duling, 1989; Johnson, 1968). Autoregulation, however, contains not only a pressure sensitive (myogenic) mechanism, but also a flow sensitive mechanism due to alterations in vasodilator metabolites during the period of altered flow (Johnson, 1978). Recent studies have revealed that in addition to metabolic effects, flow-sensitive responses involve endothelial function in which the endothelium serves as a unique mechanoreceptor, sensing and transducing physical stimuli (e.g., shear forces) into changes in vascular tone by release of endothelium-derived relaxing factor (EDRF) (Rubanyi, 1991). This endothelial-dependent, shear stress-induced response has been reported in both large and resistance arteries (Fujii *et al.*, 1991; Kuo *et al.*, 1992; Phol and Lamontagne, 1991). Accordingly, the regulation of local blood flow appears to be a complicated interaction between several regulatory mechanisms and these mechanisms may facilitate or attenuate each other. These interactions depend on the local environment, such as the characteristics of the vascular

wall and the functional or transduction pathway(s) presented in endothelial cells along particular segments of the arterioles.

There is a graded myogenic response along the arteriolar network in the hamster cheek pouch, and this myogenic response maintains a larger gain towards the distal branches of the arterioles (Davis, 1991; Jackson and Duling, 1989). However, these conclusions were based on *in vitro* experiments using isolated arterioles; thus, the influence of upstream arterioles on the response of downstream arterioles could not be assessed in these studies. Since our previous study showed that there is a heterogeneity of Ach-induced EDRF activity in series-arranged microvascular networks (Tang and Joyner, 1992), and that endothelial cells function differently in the distinct segments of series-arranged arterioles (Tang and Joyner, 1993), autoregulatory mechanisms in distinct segments of series-arranged arterioles may have different features. Hence, they may be governed by differed mechanisms or transendothelial cell pathways.

This study was designed to investigate the autoregulatory behavior and the involvement of endothelial-dependent, flow-induced dilation in the regulation of series-arranged arterioles in the hamster cheek pouch using an intact, *in situ*, pump-perfused, cell-free microvascular network. The basic hypotheses are that differential mechanisms govern local autoregulatory behavior in distinct segments of the series-arranged arterioles, and the endothelial-dependent, flow-induced dilatory mechanism is involved differently in specific arteriolar segments.

Experimental Protocols

Vasoactivity testing

To establish certain fundamental requirements/criteria for this *ex vivo* preparation, responses of selected arterioles to an endothelial-dependent vasodilator, acetylcholine (Ach, 10^{-6} M), and a receptor-mediated vasoconstrictor, norepinephrine (NE, 10^{-7} M), were analyzed. The perfusion flow was set at 2ml/min and these vasoactive substances were applied topically by adding them in the suffusate. The preparation was stabilized for 20 min prior to the testing. Then, the basal diameters of both 2nd and 4th order arterioles were recorded followed by adding either Ach or NE into the suffusate line. The diameters of these selected arterioles were recorded again during the application of these substances. The maximum dilated or constricted diameter obtained after Ach or NE application were used as the experimental diameters (D_E).

Autoregulatory behavior of large and small arterioles in the hamster cheek pouch under the control condition.

The protocol testing autoregulatory behavior of large and small arterioles under the control condition is shown in Figure 20a. After the preparation was stabilized for 20-30 minutes with a perfusion flow (Q_p) of 1 ml/min and recorded perfusion pressure (P_p), control diameters (D_C) and microsphere velocities for selected arterioles (A_2 and A_4) were recorded at this basal perfusion flow. Then, the perfusion flow, therefore the perfusion pressure, was increased stepwise from this initial level to a final Q_p level of 3ml/min in increments of 1ml/min. Each Q_p /and or P_p level was held for 1-2 min while changes in diameters (D_E) and microsphere centerline velocities (V_C) were measured in

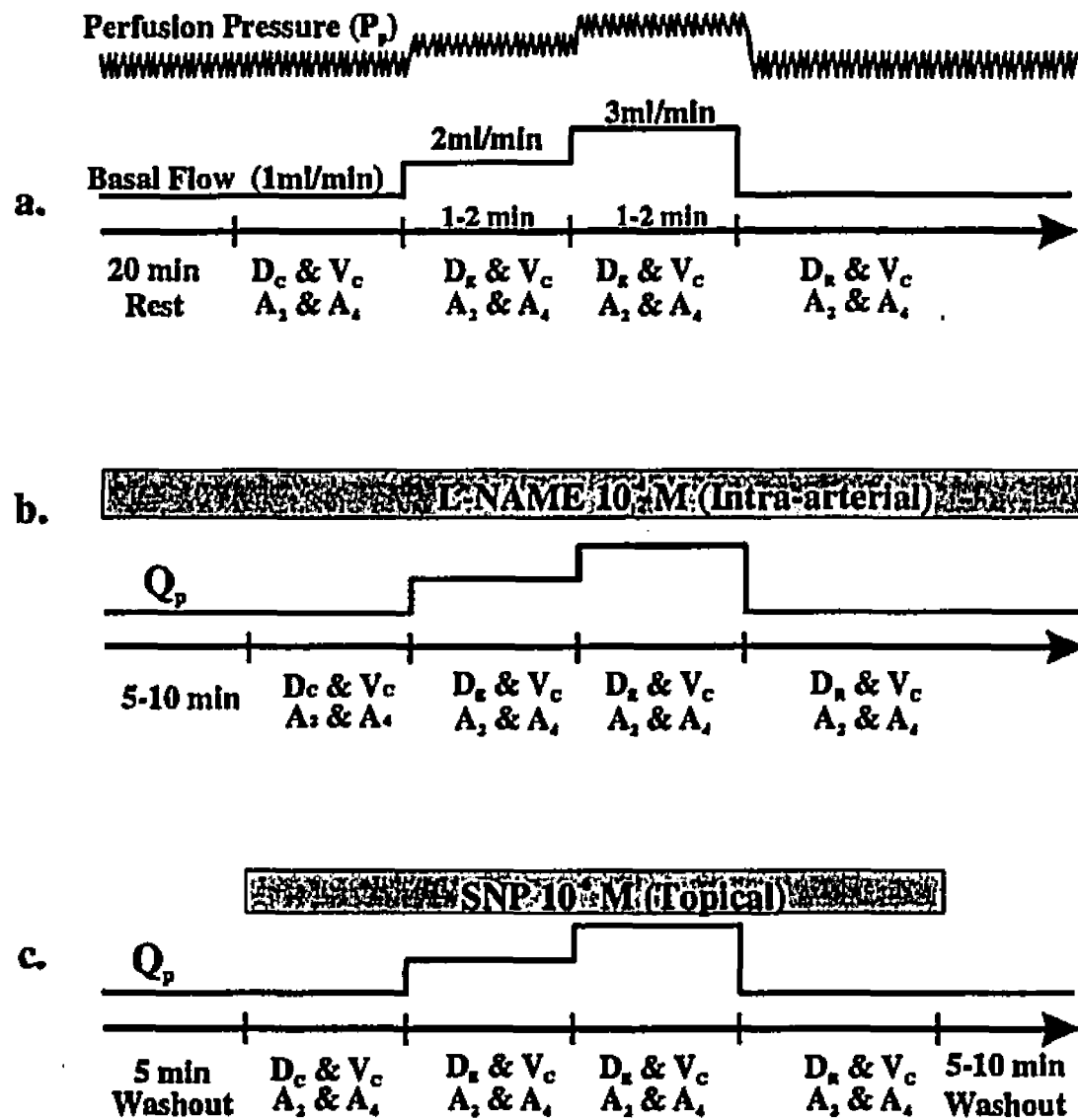
large (A_2) or small (A_4) arterioles (one at a time). At the end of this protocol, the perfusion flow and pressure were reduced to the initial level (1ml/min) and measurements on both diameters (D_R) and microsphere centerline velocities of selected arterioles (A_2 or A_4) repeated.

Role of the endothelium in mediating the autoregulatory behavior of large and small arterioles: flow-induced and myogenic regulatory responses.

After establishing the autoregulatory response under control conditions, normal perfusate was replaced with the one added with N^ω-Nitro L-Arginine Methyl Ester (L-NAME) with a final concentration of 10^{-4} M. Five-ten minute stabilization was allowed at a perfusion flow of 1 ml/min for this new perfusate. Then, the basal diameters (D_0) and microsphere centerline velocities (V_C) of A_2 and A_4 were recorded. Thereafter, the perfusion flow and pressure were increased in the same manner as described above while the changes in arteriolar diameter (D_P) and microsphere centerline velocity measured (Fig. 20b).

After the perfusate was switched back to the normal perfusion solution, the remaining L-NAME was washed out for 5-10 minutes at a basal flow rate of 1ml/min. Then, sodium nitroprusside (SNP), an endothelial-independent vasodilator, was administered into the suffusate line at a constant flow of 0.2ml/min. Thus, the final concentration of SNP in the suffusate was 10^{-6} M. After equilibration, the diameters and microsphere velocity of A_2 and A_4 were recorded in this new state and the procedure described above followed (Figure 20c).

Figure 20. Experimental protocols for testing the autoregulatory responses initiated by alterations in perfusion flow: a) under the control condition; b) under the condition that an inhibitor of EDRF/NO synthesis, N^w-Nitro L-arginine Methyl Ester (L-NAME, 10⁻⁴ M) is perfused into the vasculature; and c) under the condition that myogenic tone of the vascular smooth muscle is released by topically applying a vasodilator, sodium nitroprusside (SNP, 10⁻⁶ M). Perfusion pressure (top panel) is monitored during whole course of the experiment. Control diameter (D_c) is obtained at basal flow rate (1ml/min) and the centerline velocity of the perfusate (V_c) is measured at each perfusion flow under all of three experimental conditions. Experimental diameter (D_p) is recorded after stepwise alterations of the perfusion flow.



Results

Local volume flow, an index for determining the perfusion flow

To determine the proper perfusion flow rates (Q_p), the local volume flow (Q_m) at the level of 2nd and 4th order arterioles was calculated from microsphere centerline velocities measured at each Q_p level. These calculated values were compared with those obtained from the *in vivo* preparations. As shown in Table 7, Q_m obtained from the *in vivo* preparations was within the range of 1.86 to 2.98 nl/sec (averaged at 2.46 ± 0.2 nl/sec) in 2nd order arterioles and 0.11 to 0.25 nl/sec (averaged at 0.16 ± 0.02 nl/sec) in 4th order arterioles. In the *ex vivo*, pump-perfused preparations, the initial perfusion flow (Q_p -1 @ 1 ml/min) yielded a local volume flow of 1.35 ± 0.25 nl/sec in large arterioles (A_2) and this volume flow was significantly lower than that observed in the *in vivo* condition. In small arterioles (A_4), however, Q_m (0.15 ± 0.04 nl/sec) was not significantly different from those *in vivo* data (0.16 ± 0.02 nl/sec). At a higher perfusion flow (Q_p -2 @ 2 ml/min), Q_m obtained from the selected arterioles was similar to those *in vivo* data in both A_2 (2.82 ± 0.67 compare to 2.46 ± 0.2 (nl/sec)) and A_4 (0.19 ± 0.04 compare to 0.16 ± 0.2 (nl/sec)) (student t test, $P > 0.05$). Perfusing at a higher flow (Q_p -3 @ 3 ml/min) yielded a higher Q_m in both A_2 (5.32 ± 1.33) and A_4 (0.32 ± 0.09) as compare to the *in vivo* condition but they both were not significant different from the *in vivo* flow. Therefore, a perfusion flow of Q_p -2 (2ml/min) provided a local volume flow close to the local environment at "normal" conditions; hence, it was used to perform the vasoactive testing when evaluating this *ex vivo* preparation. Perfusion flows of Q_p -1, Q_p -2 and Q_p -3 were chosen to perform the autoregulatory response testing since they provided unique situations below, equal and slightly above this "normal" flow condition.

Data concerning volume flow in local arterioles of the normal hamster cheek pouch published by Joyner *et al.* (1979) also are listed in Table 7. These results were obtained from small, 3rd order, arterioles of the hamster cheek pouch under the *in vivo* condition by the cross-correlation technique for measuring red blood cell velocity. These data were very consistent with ours in that volume flow in 3rd order arterioles of the hamster cheek pouch was 0.52 to 0.91 nl/sec; this was somewhere between the average flow of A_2 and A_4 obtained in our present study.

Vasoactivity of the *ex vivo* preparation

To establish the vasoactive characteristics of this *ex vivo* preparation, microvascular responses to a dilator, acetylcholine, and a constrictor, norepinephrine, were tested and these data were compared with those obtained from the *in vivo* preparation described previously in Chapters 4 and 5.

As shown in Table 8, diameters of selected large arterioles (2nd order) averaged $46 \pm 3 \mu\text{m}$; while, those smaller arterioles (4th order) were $13 \pm 1 \mu\text{m}$. Addition of acetylcholine (10^{-6}M) in the suffusate produced a $12 \pm 2\%$ and $44 \pm 9\%$ dilation in 2nd and 4th order arterioles, respectively. Norepinephrine, however, produced a $33 \pm 7\%$ constriction (as indicated by negative numbers) in large arterioles and $26 \pm 5\%$ constriction in small arterioles. These vasoactive responses were comparable to those measured using the *in vivo* preparation and no significant differences were found, by analysis of variance, between two different preparations.

Table 7. Comparison of microvascular volume flow for different orders of cheek pouch arterioles obtained from *in vivo* and *ex vivo* preparations.

Vessel Order	Basal Diameter (μm)	Microvascular Volume Flow ^a			
		<i>in vivo</i>	<i>ex vivo</i> measured at different Q_p levels		
			Q_p -1 ^b	Q_p -2 ^b	Q_p -3 ^b
2nd order	52 ± 4 (5)	2.46 ± 0.20			
	47 ± 2 (6)		1.35 $\pm 0.25^\dagger$	2.82 ± 0.67	5.32 ± 1.33
3rd order ^c	30-40	0.52-0.91			
4th order	14 ± 1 (6)	0.16 ± 0.02			
	17 ± 1 (6)		0.15 ± 0.04	0.19 ± 0.04	0.32 ± 0.09

Note. Numbers in parentheses indicate numbers of the experiments.

a Microvascular volume flow, nl/sec.

b Perfusion flow (Q_p) set by the perfusion pump: 1, 2 and 3 ml/min respectively.

c Data from Joyner *et al.* (1979).

[†] Significant different ($P < 0.05$) compare to those obtained from *in vivo* preparations by student t test.

Table 8. Comparison of microvascular responses to acetylcholine (Ach) and norepinephrine (NE) for *ex vivo*, pump-perfused, and *in vivo*, autoperfused, preparations of the hamster cheek pouch.

Arterioles	<i>Ex vivo</i> ^a			<i>In vivo</i>		
	D _c (μm)	Ach (%ΔD _c)	NE (%ΔD _c)	D _c (μm)	Ach (%ΔD _c)	NE (%ΔD _c)
2nd order	46 ±3 (6)	12 ±2	-33 ±7	42 ±2 (5)	19 ±5	-25 ±4
4th order	13 ±1 (6)	44 ±9	-26 ±5	14 ±1 (5)	54 ±13	-23 ±3

a *Ex vivo*: perfused at a flow of 2 ml/min.

Data are expressed as percentage change in control diameter (%ΔD_c); the values are means with standard errors. Numbers in parentheses indicate numbers of the experiments.

Negative numbers indicate vasoconstriction; while positive numbers indicate vasodilation.

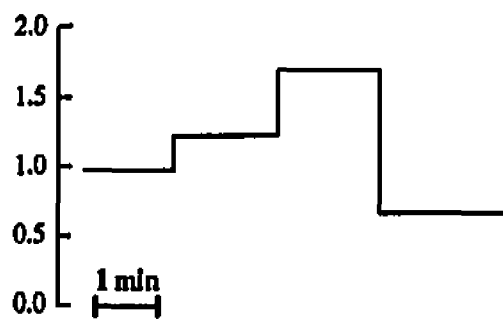
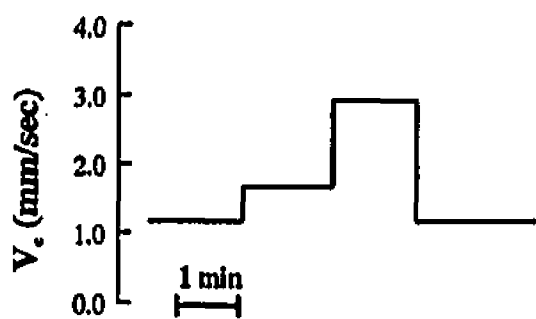
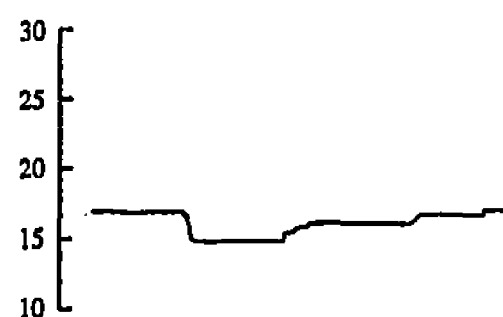
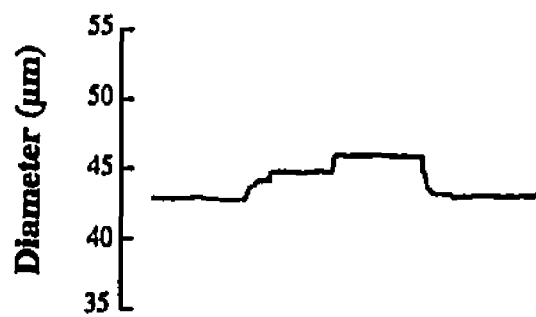
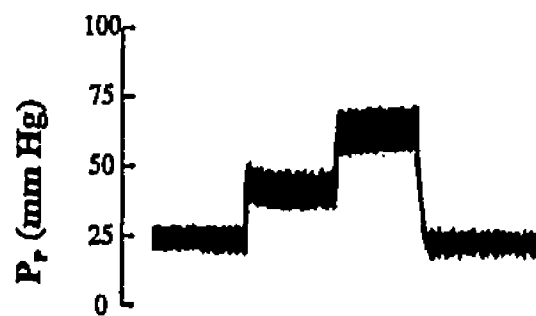
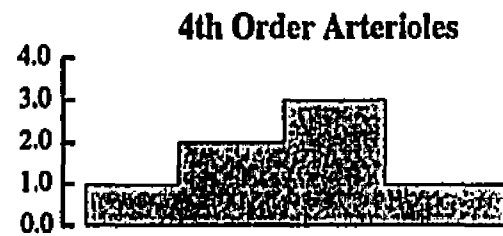
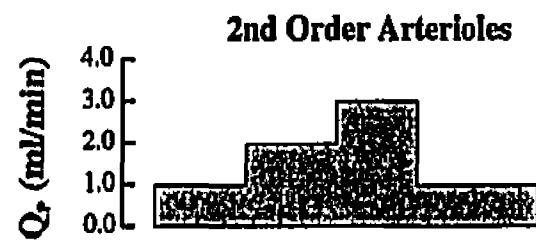
Ach was 10⁻⁶ M; NE was 10⁻⁷ M. No significant differences were found between two groups by the analysis of variance (P>0.05).

Autoregulatory characteristics of arterioles in the hamster cheek pouch

The autoregulatory behavior of cheek pouch arterioles was investigated by measuring changes in arteriolar diameter, microsphere centerline velocity (V_c , averaged at each Q_p level) and perfusion pressure (P_p) while stepwise increasing the perfusion flow in increments of 1 ml/min. Records from one typical experiment are shown in Figure 21. As shown in the top two panels, stepwise alterations in perfusion flow (Q_p) were associated with proportional elevations of perfusion pressure also in a stepwise fashion. In large, 2nd order, arterioles (left panels), elevations in Q_p resulted in a vasodilatation, indicated by stepwise increases in arteriolar diameter, and an increase in centerline velocity under these control conditions. When Q_p was returned to the initial level, the diameter and velocity of this arteriole recovered to their basal values. In small, 4th order arterioles (right panels), on the other hand, the first step Q_p elevation produced a reduction in arteriolar diameter with an increase in centerline velocity. Nevertheless, a further Q_p elevation (Q_{p-3} @ 3 ml/min) led to an increase in arteriolar diameter which returned it towards to its basal level; there also was a substantial increase in centerline velocity. When Q_p was returned to the initial level, the diameter of this small arteriole recovered to its basal level; whereas, the flow velocity in this vessel recovered somewhat over the basal level.

Table 9 summarizes these parameters measured during alterations of perfusion flow including changes in perfusion pressure (P_p), control/basal diameter (diameter at initial Q_p level), changes in diameter (normalized as a percentage of the control diameter, $\%D_c$) and centerline velocity (V_c) in large (A_2) and small (A_4) arterioles under these control conditions. The perfusion pressure at initial perfusion flow (Q_{p-1} , 1 ml/min)

Figure 21. Recorded time courses of alterations in perfusion pressure (P_p , 2nd panel), arteriolar diameter (3rd panel), and averaged centerline velocity (V_c , bottom panel) after alterations in perfusion flow (top panel) under control conditions in both 2nd (left) and 4th (right) order arterioles.



averaged 40 ± 1 mmHg and it remained at a similar level (41 ± 1 mmHg, $P > 0.05$) when returning Q_p from the higher level to the initial level (Q_p -R). Stepwise elevations in perfusion flow produced proportional elevations in perfusion pressure, 64 ± 2 mmHg after the first step and 89 ± 3 mmHg after the second step elevation in Q_p . Hence, each step-change in perfusion flow produced a similar ΔP of 24–25 mmHg.

Autoregulatory behavior was manifested by diameter changes in response to altered perfusion flow/or pressure. In large (A_2) arterioles, arteriolar diameters averaged 47 ± 2 μ m at initial perfusion flow (Q_p -1) and they were increased to $105 \pm 1\%$ and $109 \pm 1\%$ of this control at perfusion flow of 2 ml/min (Q_p -2) and 3 ml/min (Q_p -3), indicating a flow-induced dilation. In small (A_4) arterioles, on the other hand, basal diameters measured at Q_p -1 averaged 17 ± 1 μ m; whereas, first step elevation in perfusion flow (Q_p -2) yielded a reduction in vascular diameter with a normalized diameter of $93 \pm 1\%$. Raising perfusion flow further to the level of Q_p -3, however, resulted in a slight dilation that gave a greater values of $\%D_c$ ($98 \pm 3\%$) compare to those at Q_p -2. When comparing these responses to those observed in large arterioles by paired t test, significant differences were found at both two higher Q_p levels ($P < 0.05$). After returning perfusion flow to the initial level (Q_p -R), diameters in either order of arterioles recovered to their basal level.

Centerline velocities (V_c), measured using microspheres as tracers, also are listed in Table 9. In large (A_2) arterioles, V_c values obtained at Q_p -1 were 1.24 ± 0.2 mm/sec, similar values were obtained at Q_p -R (1.30 ± 0.2 mm/sec, $P > 0.05$ by paired t test). Increasing perfusion flow to Q_p -2 and Q_p -3 increased V_c values to 2.28 ± 0.4 mm/sec and 3.85 ± 0.7 mm/sec, respectively. In small (A_4) arterioles, V_c values were lower than those

Table 9. Summary of the parameters measured during alterations of perfusion flow, including perfusion pressure, vascular diameter and centerline velocity, in large, 2nd order (A_2) and small, 4th order (A_4) arterioles under control condition.

Measured Parameters		Perfusion Flow			
		Q_p-1^a	Q_p-2^a	Q_p-3^a	Q_p-R^a
P_p^b (n=6)		40 ± 1	64 ± 2	89 ± 3	41 ± 1
Arteriolar Diameter ^c (n=6)	A_2	47 ± 2	105% ± 1	109% ± 1	101% ± 2
	A_4	17 ± 1	93% ± 1	98% ± 1	100% ± 1
V_c^d (n=6)	A_2	1.24 ± 0.2	2.28 ± 0.4	3.85 ± 0.7	1.30 ± 0.2
	A_4	0.95 ± 0.2	1.43 ± 0.3	2.08 ± 0.6	0.76 ± 0.2

- a Perfusion flow (Q_p) set by the perfusion pump: 1, 2 and 3 ml/min respectively.
 Q_p-R is the perfusion flow that returned to the initial level (1 ml/min) from the level of Q_p-3 .
- b Perfusion pressure (mmHg).
- c Diameters at Q_p-1 are basal/control diameters (D_c , μm); whereas, diameters at Q_p-2 , Q_p-3 and Q_p-R were normalized as $\%D_c$.
- d Centerline velocity (mm/sec) measured using microspheres as tracers.
 Numbers in parentheses indicate numbers of the experiments.

observed in A_2 at all Q_p levels; but all the differences are not significant. Similar to those seen in the large arterioles, V_c values were proportionally increased by step changes in perfusion flow in these small arterioles and they returned to their basal level (measured at Q_{p-1}) at Q_p -R. These V_c values are the basis for the calculation of the local volume flow at each Q_p level.

Involvement of the endothelium, the EDRF/NO pathway in microvascular autoregulation as indicated by the effect of L-NAME

Records from the same experiment as depicted in Figure 21 but under the condition of blocking EDRF/NO release by applying L-NAME (10^{-4} M) intra-arterially are shown in Figure 22. Unlike the responses in the control condition, stepwise elevations in Q_p produced constriction in 2nd order arterioles in a Q_p -dependent manner as illustrated by a stepwise decrease in arteriolar diameter. However, the centerline velocity in this vessel remained elevated in response to Q_p elevations in a similar manner as seen in the control condition (left panels). In the 4th order arteriole (right panels), constriction was observed upon the first step Q_p elevation and additional constriction occurred with the further elevation of Q_p . Centerline velocity in this vessel continuously increased similar as seen in the control condition.

Summary data obtained under this condition can be found in Table 10. The perfusion pressures were slightly higher than those obtained under control conditions, but no significant differences were found at any Q_p level ($P > 0.05$ by paired t test); therefore, ΔP (26 mmHg) generated by each step Q_p elevation was not significantly altered. Diameter responses, on the other hand, displayed a different pattern under this condition. In large arterioles (A_2), increases in perfusion flow produced vasoconstriction, as

Figure 22. Recorded time courses of alterations in perfusion pressure (P_p , 2nd panel), arteriolar diameter (3rd panel), and averaged centerline velocity (V_c , bottom panel) in both 2nd (left) and 4th (right) order arterioles in response to changes in perfusion flow (top panel) under the condition of inhibiting of EDRF/NO release by with L-NAME (10^{-4} M).

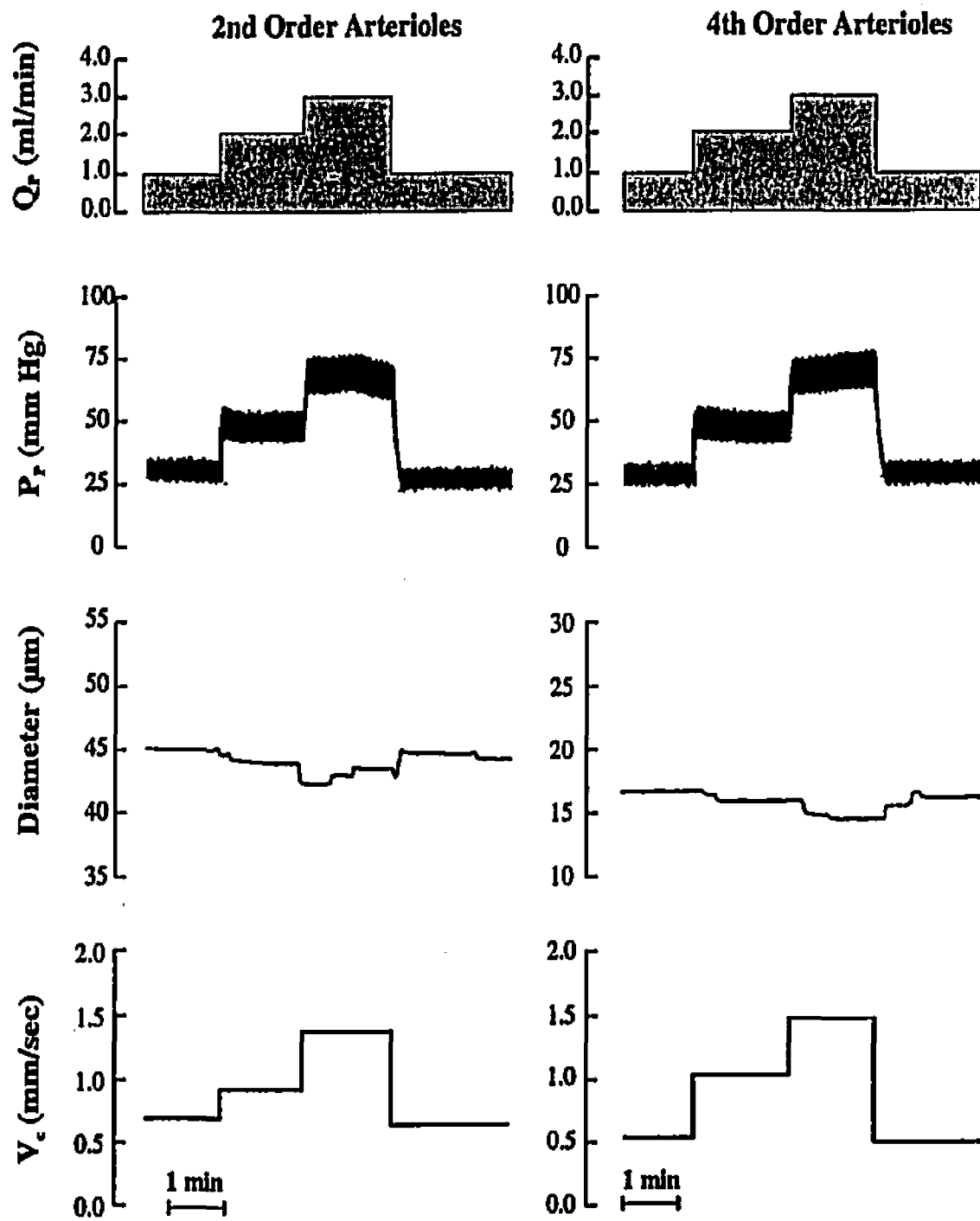


Table 10. Summary of the parameters measured during alterations of perfusion flow, including perfusion pressure, vascular diameter and centerline velocity, in large, 2nd order (A_2) and small, 4th order (A_4) arterioles in the condition of blocking EDRF/NO synthesis by L-NAME (10^{-4} M) perfusion.

Measured Parameters		Perfusion Flow			
		Q_p-1^a	Q_p-2^a	Q_p-3^a	Q_p-R^a
P_p^b		44 ± 5	70 ± 6	96 ± 8	45 ± 5
(n=6)					
Arteriolar Diameter ^c	A_2	48 ± 2	96% ± 1	93% ± 1	98% ± 1
(n=6)	A_4	16 ± 1	93% ± 2	86% ± 2	97% ± 1
V_c^d	A_2	1.34 ± 0.3	2.39 ± 0.6	3.55 ± 0.8	1.30 ± 0.2
(n=6)	A_4	0.79 ± 0.2	1.57 ± 0.4	2.47 ± 0.7	0.84 ± 0.2

- a Perfusion flow (Q_p) set by the perfusion pump: 1, 2 and 3 ml/min respectively. Q_p-R is the perfusion flow that returned to the initial level (1 ml/min) from the level of Q_p-3 .
- b Perfusion pressure (mmHg).
- c Diameters at Q_p-1 are basal/control diameters (D_c , μm); whereas, diameters at Q_p-2 , Q_p-3 and Q_p-R were normalized as $\%D_c$.
- d Centerline velocity (mm/sec) measured using microspheres as tracers. Numbers in parentheses indicate numbers of the experiments.

indicated by reductions in arteriolar diameter, in a flow-dependent manner ($\%D_c$ averaged $96\pm1\%$ and $93\pm1\%$ at Q_p -2 and Q_p -3); and these responses were significantly different from those seen in the control condition ($P<0.05$). Furthermore, these arterioles recovered towards their basal diameter at Q_p -R. In small arterioles (A_4), arteriolar diameters reduced to a similar degree ($93\pm2\%$) as seen in control condition at Q_p -2. However, additional constriction was observed, as indicated by a further reduction in $\%D_c$ value ($86\pm2\%$), when higher Q_p level (Q_p -3) was reached, and this $\%D_c$ value was significantly different compare to that obtained in the control condition at same Q_p level ($P<0.05$ by paired t test). Centerline velocity under this condition altered in a similar pattern as seen in the control in response to alterations in perfusion flow.

Passive responses after uncoupling microvascular tone by topical application of SNP

Records from this same experiment but under the condition of uncoupling microvascular tone by SNP (10^{-6} M) are shown in Figure 23. Topical application of SNP brought basal diameters of both large, 2nd order and small, 4th order arterioles up to a new D_c level ($48\text{ }\mu\text{m}$ for A_2 and $22\text{ }\mu\text{m}$ for A_4 in this case). When perfusion flow was stepwise increased, the large, 2nd order arteriole dilated in a stepwise manner to a degree greater than those seen in the control condition. Likewise, in small, 4th order arterioles, step changes in Q_p produced a continued dilation. These arterioles recovered to their basal diameters when returning perfusion flow to the initial level. Furthermore, in both orders of arterioles, centerline velocity increased dramatically by the first step Q_p elevation; and further increase in V_c was observed following the 2nd step Q_p elevation.

Summary data obtained under this condition are gathered in Table 11. Notice that after the topical application of SNP, the basal diameter measured at Q_p -1 increased

Figure 23. Recorded time courses of alterations in perfusion pressure (P_p , 2nd panel), arteriolar diameter (3rd panel), and averaged centerline velocity (V_c , bottom panel) in both 2nd (left) and 4th (right) order arterioles in response to changes in perfusion flow after relaxing vascular smooth muscle with sodium nitroprusside (SNP, 10^{-6} M).

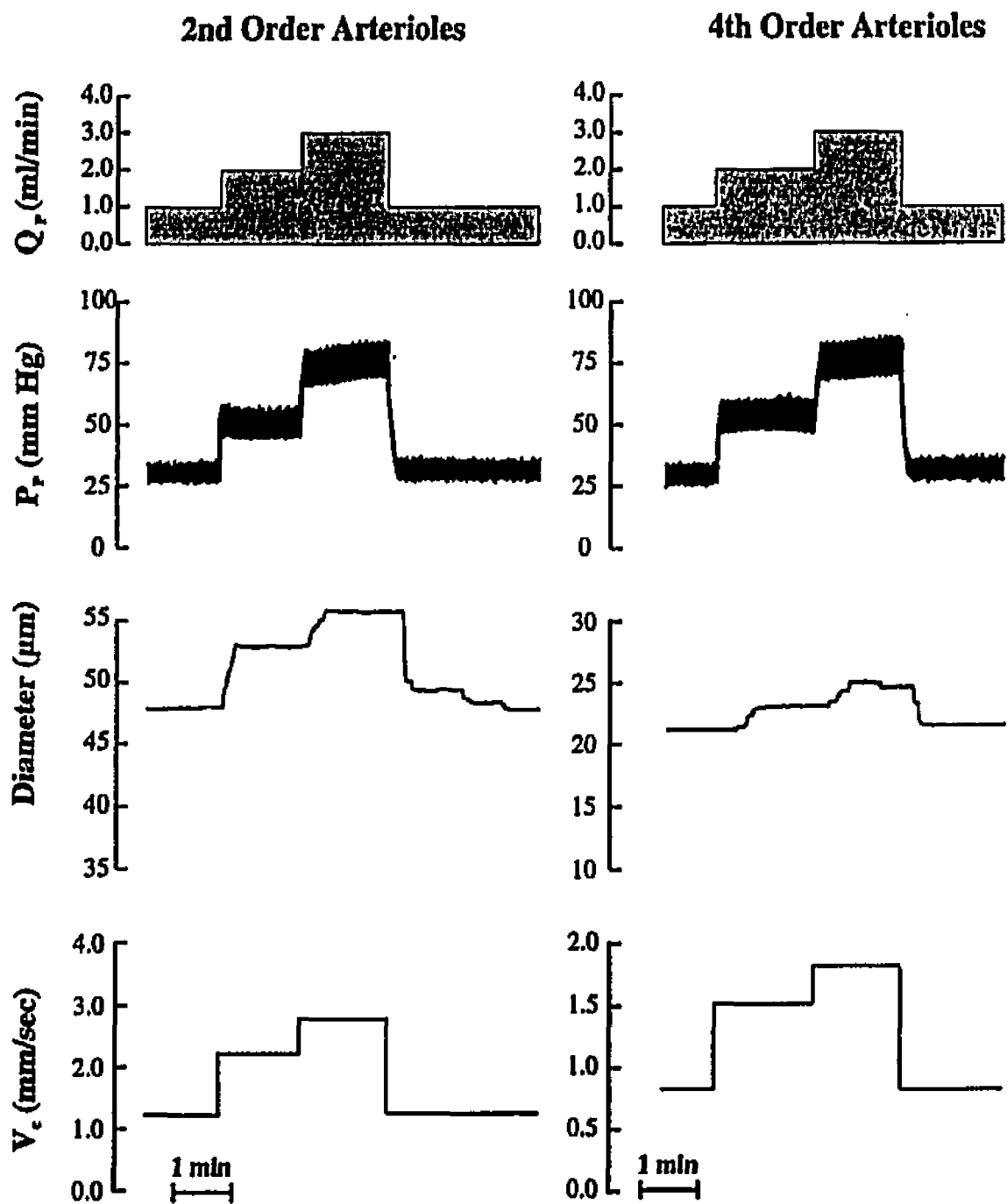


Table 11. Summary of the parameters measured during alterations of perfusion flow, including perfusion pressure, vascular diameter and centerline velocity, in large, 2nd order (A_2) and small, 4th order (A_4) arterioles in the condition of uncoupling myogenic tone by topical application of SNP (10^{-6} M).

Measured Parameters		Perfusion Flow			
		Q_p-1^a	Q_p-2^a	Q_p-3^a	Q_p-R^a
P_p^b (n=6)		43 ± 3	68 ± 4	92 ± 5	44 ± 3
Arteriolar Diameter ^c (n=6)	A_2	52 ± 2	106% ± 1	112% ± 1	101% ± 1
	A_4	19 ± 1	108% ± 2	115% ± 3	99% ± 1
V_c^d (n=6)	A_2	1.46 ± 0.3	2.65 ± 0.6	4.02 ± 1.1	1.58 ± 0.4
	A_4	0.92 ± 0.1	1.83 ± 0.5	2.46 ± 0.7	0.99 ± 0.2

- a Perfusion flow (Q_p) set by the perfusion pump: 1, 2 and 3 ml/min respectively. Q_p-R is the perfusion flow that returned to the initial level (1 ml/min) from the level of Q_p-3 .
- b Perfusion pressure (mmHg).
- c Diameters at Q_p-1 are control/basal diameters (D_c , μm); whereas, diameters at Q_p-2 , Q_p-3 and Q_p-R were normalized as $\%D_c$.
- d Centerline velocity (mm/sec) measured using microspheres as tracers. Numbers in the parenthesis indicate numbers of the experiments.

12±2% in large arterioles (A_2) and 24±2% in small arterioles (A_4). The perfusion pressures were not significantly altered at any Q_p level compare to those obtained in the control condition and the pressure gradient (ΔP) generated by each step elevation of perfusion flow in this condition was 24–25 mmHg ($P>0.05$ compare to the control condition). In large (A_2) arterioles, stepwise elevations of perfusion flow produced proportional increases in diameter, as indicated by same amount increases in $\%D_c$ at each higher Q_p level (106±1% at Q_p -2, and 112±1% at Q_p -3), and dilation seen at Q_p -3 were significantly greater than those seen in the control condition ($P<0.05$ by paired t test). In small (A_4) arterioles, responses were contradictory to those observed in the control condition. Stepwise increases in perfusion flow produced dilation in these small arterioles to 108±2% (at Q_p -2) and 115±1% (at Q_p -3) of their control diameters; and these responses were significantly different ($P<0.001$) from the control condition which displayed an overall constriction. Centerline velocities obtained under this condition were altered in a similar pattern as seen in the control in which V_c increased in a Q_p dependent manner and it recovered to its basal level when Q_p was returned back to the initial level. Diameters of both large and small arterioles recovered to their basal level at Q_p -R.

Differential features of autoregulation: Interaction between myogenic responses and flow-induced dilation in large and small arterioles

To analyze the autoregulatory responses of these arterioles, diameter changes, characterized as normalized diameter ($\%D_c$), were plotted as a function of the perfusion pressures (P_p), which altered proportionally to the changes in perfusion flow (Q_p), under conditions of control (empty circles), after inhibition of EDRF/NO synthesis (solid

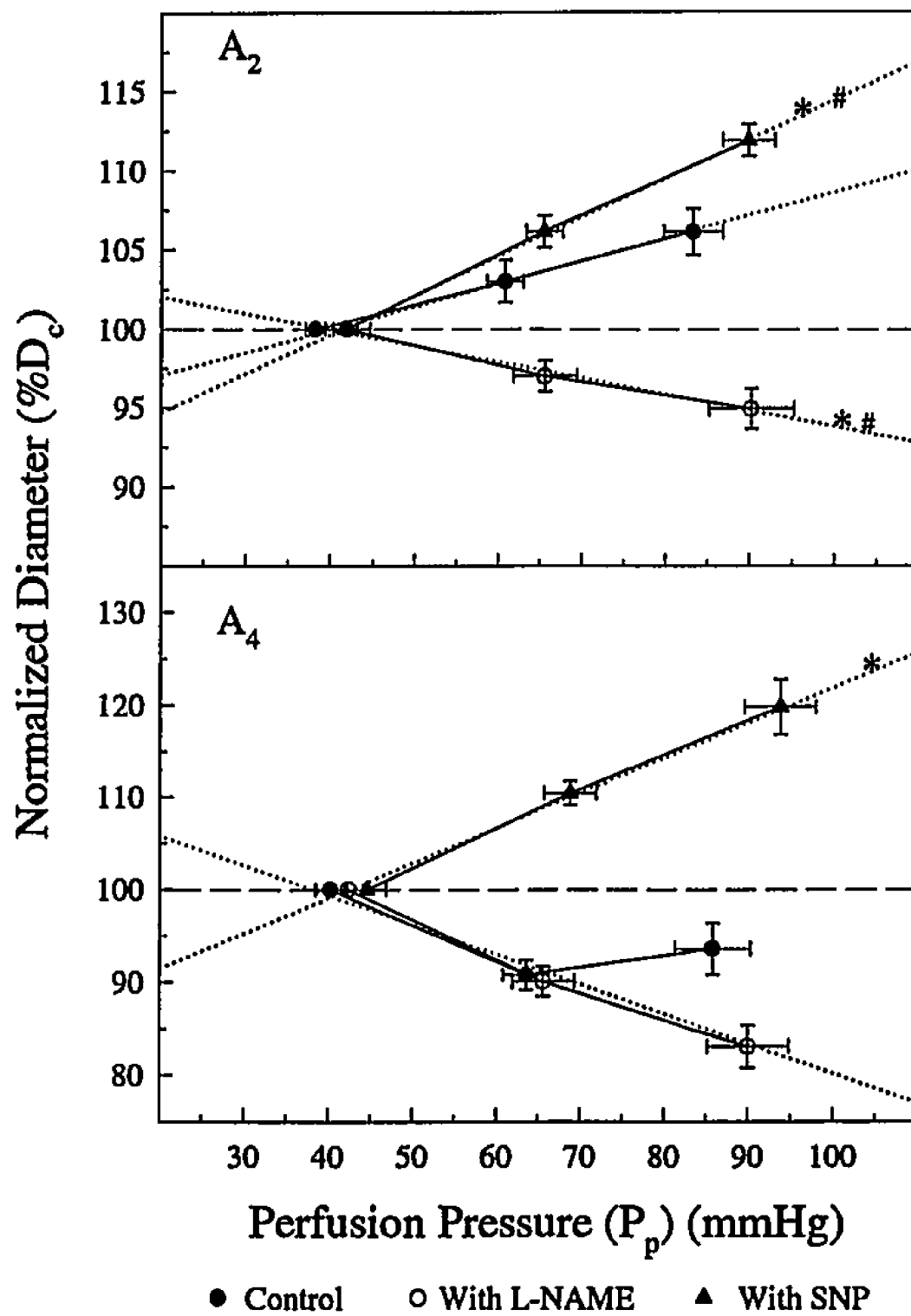
circles), and after uncoupling microvascular tone with SNP (triangles, Figure 24). The common regression lines combined from 12 experiments for each condition also were plotted by dotted lines.

Under control conditions, the diameter-pressure ($\%D_c-P_p$) curves for large, 2nd order arterioles (A_2) were linear ($r=0.98$) and their common regression line had a significant positive correlation with a slope of $+0.14$ (top panel, empty circles). For the small, 4th order arterioles (A_4), however, the diameter-pressure curves were not linear. In fact as the perfusion pressure further increased, arteriolar diameter decrease remained at this level; therefore, the correlation for their common regression line was not significant (bottom panel, empty circles). When comparing slopes of common regression lines for large versus small arterioles under this control condition, a significant difference was evident ($P<0.05$).

After the synthesis/release of EDRF/NO had been inhibited with L-NAME (solid circles), there still was a linear relation in the $\%D_c-P_p$ curves ($r=0.99$) in 2nd order arterioles (top panel) but the slope of their common regression line became negative (-0.10). Comparison of slopes in large, 2nd order, arterioles indicates a significant difference between control and EDRF/NO blocked condition ($P<0.05$). In small, 4th order, arterioles (bottom panel), application of L-NAME linearized ($r=0.98$) the $\%D_c-P_p$ curves and the correlation of their common regression line became significant with a negative slope of -0.32 . This slope had a significantly higher value (disregard the negative sign) compare to large arterioles ($P<0.001$ by two way analysis of variance).

When SNP was added to these microvessels (triangles), both large (A_2) and small (A_4) arterioles displayed a linear correlation in the $\%D_c-P_p$ curves ($r=0.999$ and 0.997 ,

Figure 24. Relationships between the perfusion pressure (P_p) and changes in diameter, characterized as a normalized diameter ($\%D_0$), in the condition of control (open circles); after EDRF/NO blockade by L-NAME (10^{-4} M) (solid circles); and after uncoupling the myogenic tone by the topical application of SNP (10^{-6} M, triangles) in both 2nd (top panel) and 4th (bottom panel) order arterioles. Basal diameter level is plotted by dashed line and the common regression lines combined from 12 experiments in different conditions are also plotted by dotted lines. Data shown are means with their standard errors. *: slope of combined regression line significantly differed from that seen under control condition. #: Slope of the combined regression line for 2nd order arterioles significantly differed from that of 4th order arterioles under the same experimental condition.



respectively) and the slopes of their common regression lines were positive (+0.14, +0.38, respectively). These slopes were significantly different from those obtained in both control and L-NAME perfused conditions ($P < 0.05$) in either 2nd order or 4th order arterioles. Also, the common regression lines for the large versus the small arterioles were significantly different ($P < 0.05$) with the slope for the large arterioles being smaller than that for the small arterioles.

Table 12 summarizes the common slopes of regressed $\%D_c-P_p$ relations obtained under three different conditions in both large (A_2) and small (A_4) arterioles. In large, 2nd order arterioles, statistical differences were found in slopes between passive response (with SNP) and control response as well as between control and response after EDRF blockade (with L-NAME). In small, 4th order arterioles, the slope of control response was significantly different from that of passive response. Inhibition of EDRF synthesis with L-NAME had no significant effect on the slope of control response. In all three experimental conditions, the slopes of the $\%D_c-P_p$ relation in large, 2nd order arterioles were significantly different from those of small, 4th order arterioles.

Correlations between perfusion flow and local volume flow, shear stress gradient experienced by large and small arterioles

To compare relative changes in local volume flow (Q_m), created by each step alteration of perfusion flow (Q_p), in large (A_2) and small (A_4) arterioles, Q_m was calculated from the combinations of centerline velocities and diameters of the selected arterioles. These Q_m values were averaged at each Q_p level ($n=6$) and they were plotted as a function of Q_p for control, L-NAME perfused and SNP suffused conditions.

As shown in Figure 25, in control condition (open circles), Q_m-Q_p relations in both

Table 12. Summary of slopes of combined regression lines of %D_C-P_p relations^a in large (A₂) and small (A₄) arterioles under different experimental conditions.

		Experimental Conditions		
		Control	L-NAME ^b	SNP ^c
%D _C -P _p Slopes	A ₂	0.14	-0.10*	0.25*
	A ₄	-0.13	-0.32	0.38*
Comparison between A ₂ and A ₄		#	#	#

a Slopes are common slopes combined from 12 experiments and are compared by two way analysis of variance and LSD (Ott, 1988).

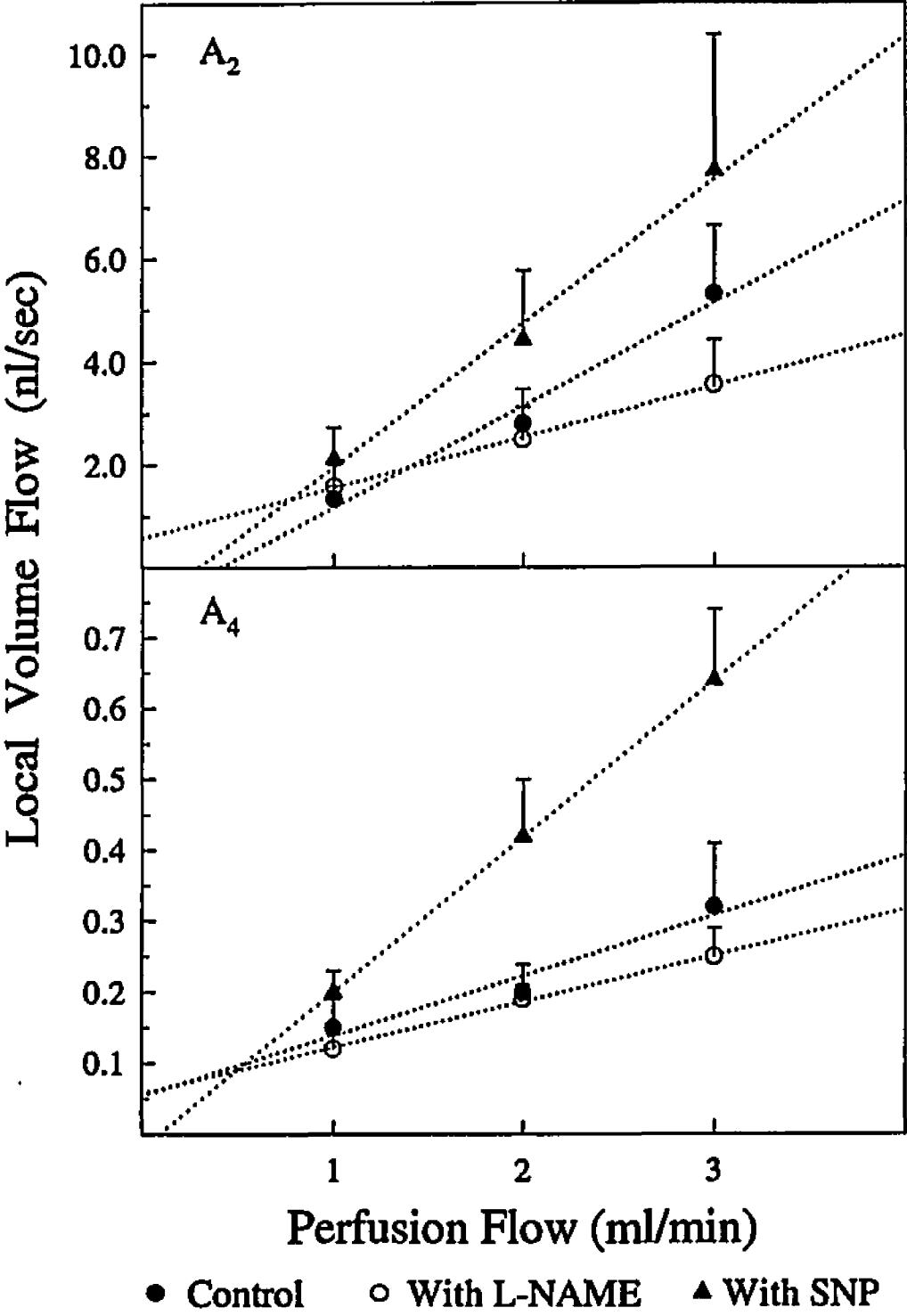
b Arterioles were perfused intra-arterially with N^ω-Nitro Arginine Methyl Ester (L-NAME, 10⁻⁴ M).

c Arterioles were suffused topically with sodium nitroprusside (SNP, 10⁻⁶ M).

* Significantly different from the control condition (P<0.05).

The differences were Significant (P<0.05).

Figure 25. Relationships between microvascular local volume flow (Q_m) in 2nd and 4th order arterioles and perfusion flow (Q_p) under conditions of: a) normal (open circles); b) L-NAME perfusion (solid circles); and c) SNP suffusion (triangles).



large (A_2 , top panel) and small arterioles (A_4 , bottom panel) were linear ($r=0.99$ in A_2 and $r=0.98$ in A_4) and the relations between Q_m and Q_p were positively correlated in either arterioles as indicated by positive slopes (1.99 in A_2 and 0.08 in A_4). In L-NAME perfused condition (solid circles), for either large or small arterioles, Q_m - Q_p relations displayed a similar pattern as those seen in the control condition and no statistical differences were found for slopes obtained in these two conditions. In SNP suffused condition (triangles), Q_m - Q_p relations were still linear in both 2nd and 4th order arterioles ($r=0.998$, $r=1.0$ respectively); whereas, the slope in 4th order arterioles was significantly increased compared to the control condition.

Slopes of these regressed lines and the comparisons between slopes were summarized in Table 13. In 2nd order arterioles, slope of Q_m - Q_p relation was 1.0 in L-NAME perfused condition and 2.79 in SNP suffused condition which both were not significant different from that of the control (1.99). In 4th order arterioles, the slope in control condition was 0.08. Perfusion of L-NAME has no significant effect on this slope (0.07); whereas, suffusion of SNP significantly raised this slope (0.22, $P<0.05$).

In fact, the slope of Q_m - Q_p relation reflects a Q_m gradient generated by each step change in Q_p ; it is this Q_m gradient that in turn produces a shear stress gradient on the vascular wall. According to Berne and Levy (1988), shear stress (τ) is positively related to volume flow (Q) but inversely related to the 3rd power of the vessel radius (r) ($\tau=4\eta Q/\pi r^3$, where η is the viscosity of the fluid flowing through). Therefore, the shear stress gradient per step change in Q_p ($\Delta\tau$) can be calculated from the combination of Q_m gradient, which is the slope of Q_m - Q_p relation, and the diameter ($d=2r$) of the vessel. As

Table 13. Comparison of Q_m - Q_p slopes and the shear stress gradients (ΔT), created by each step change in Q_p , in 2nd (A_2) and 4th (A_4) order arterioles.

		Control	With L-NAME ^a	with SNP ^b
Slopes	A_2	1.99	1.00	2.79
	A_4	0.08 [†]	0.07	0.22 [*]
Ratio ^c ($\Delta T_{A_2}/\Delta T_{A_4}$)		0.69	0.37	0.44

Slopes are compared by two way analysis of variance and LSD test.

* Significant different compare to the control.

† Significant different compare to A_2 .

a N^ω-Nitro Arginine Methyl Ester (10^{-4} M)

b Sodium Nitroprusside (10^{-6} M)

c Ratio of shear stress gradients per step change in Q_p between A_2 and A_4 .

shown in Table 13, Q_m gradients or the slope of Q_m - Q_p relations are much greater in A_2 compare to A_4 under all conditions. However, the shear stress gradients per step change in Q_p are actually higher in A_4 since the diameter of these arterioles is much smaller than those large arterioles. To cancel the effect of the viscosity η , ratios of shear stress gradients per step change in Q_p between A_2 and A_4 ($\Delta T_{A2}/\Delta T_{A4}$) were calculated and these values are listed also in Table 13. In all three experimental conditions, A_2 experienced lower ΔT compare to A_4 indicated by $\Delta T_{A2}/\Delta T_{A4}$ ratios less than one.

Discussion and Conclusions

This study provided a unique model to investigate autoregulatory mechanisms in different segments of arterioles in an intact, *in situ*, microvascular bed without the influences of metabolic responses, blood borne hormones, and neural control or sympathetic reflexes. Four major findings can be outlined from this study. First of all, both segments of arterioles (A_2 and A_4) in the hamster cheek pouch exhibit autoregulatory behavior; however, their dominant controlling mechanisms are different. Secondly, in response to elevated perfusion flow/or pressure, the primary regulating mechanism in large arterioles (A_2) is a potential endothelial-dependent, flow-induced dilation; whereas, the principal regulating element in downstream, small arterioles (A_4) is a potential endothelial-independent myogenic constriction. Thirdly, EDRF/NO produced from L-Arginine by endothelial cells is most likely the mediator responsible for the flow-induced responses for the large arterioles. Lastly, these regulatory mechanisms are independent of any blood borne hormones, cells or humoral agents. To appreciate these findings and to provide an outlook for these results, crucial aspects will be discussed including evaluation of the preparation, the relation of our results to other studies and the

implications of these findings.

Selected criteria for stable, "normal" *ex vivo* preparations

In this *ex vivo* preparation, the entire cheek pouch is perfused without directly handling individual vessels; therefore, the interruption of connections between arteriolar segments and/or damages on these vessels can be minimized. As a consequence, the intrinsic vascular tone and the normal vasoactivity has been well conserved. As shown in Table 8, at "normal" perfusion flows of 2 ml/min, which produces similar local volume flow compared to those obtained by the *in vivo* preparation, vasoactive responses to Ach and NE in arterioles (A_2 and A_4) are similar to those obtained by the *in vivo* preparation. Analysis of variance showed no significant differences between these two groups. The dilation of these arterioles to Ach without pharmacological pre-constriction indicated the presence of intrinsic vascular tone; whereas, the unaltered vasoactive responses to both dilator (Ach) and constrictor (NE) compared to those seen under *in vivo* condition evidenced a regular vasoactivity. It is particularly important to consider the intrinsic vascular tone and the normal vasoactivity as the criteria for a "normal" preparation since different results have been reported between preparations with or without the intrinsic tone. In the study of Bevan and Joyce (1990), vascular tone of the isolated rabbit ear artery was induced pharmacologically. They found that these arteries constricted to an increase in flow at low levels of vascular tone but dilated at high levels of vascular tone. When using a system that preserved intrinsic vascular tone, opposite results have been reported in which flow caused constriction when vascular tone was high but dilation when vascular tone was low (Garcia-Roldan and Bevan, 1990). Furthermore, the myogenic responses in coronary arterioles have been reported to be endothelium-

independent in studies using preparations with intrinsic tone (Kuo *et al.*, 1990a); whereas, the myogenic responses in pharmacological pre-constricted, isolated canine carotid arteries were found to be endothelium-dependent (Rubanyi, 1988). There may be other factors involved in these controversies; nevertheless, preparations that possess intrinsic vascular tone provide data with better relevancy to normal physiological situations.

On the other hand, vasoactivity, the degree of vasoactive responses to dilators or constrictors, reflects the level of active tone developed under the resting condition (Bevan and Joyce, 1990). Therefore, a "normal" vasoactivity provides a basal vascular tone comparable to those set by *in vivo* conditions, i.e. vascular smooth muscle cells are operating at a position, on their length tension curves, similar to where they act under *in vivo* conditions.

Pressure gradients and their local distribution

Data from Tables 9, 10 and 11 illustrated that the perfusion pressure gradients (ΔP) produced by each step change in perfusion flow were statistically identical under all three experimental conditions. This is important in regard to the degree of myogenic stimuli in different experimental conditions. In other words, the significant differences in autoregulatory responses seen in different conditions were not the consequences of alterations in the magnitude of myogenic stimuli.

The other concern would be the local pressure gradients experienced in these different segments of the arterioles (A_2 or A_4), i.e., at local areas, did A_2 and A_4 undergo a similar pressure gradient when the perfusion pressure was stepwise altered? According to Joyner *et al.*(1981), in normotensive hamster cheek pouches, the pressure measured in

2nd order arterioles was 57 ± 2 mmHg, 50% of the systemic pressure (113 ± 4 mmHg); whereas, the pressure obtained in 4th order arterioles was 31 ± 1 mmHg, 27% of the systemic pressure. Meanwhile, their data obtained from hypertensive hamsters (systemic pressure 133 ± 3 mmHg) showed that when the systemic pressures increased (20 mmHg higher compare to normotensive animals), local pressures in 2nd and 4th order arterioles also were elevated in a proportional manner. The pressure was 65 ± 2 mmHg in A_2 and 37 ± 3 mmHg in A_4 , which still accounted 49% and 28% of the systemic pressure.

Similar data also can be found in another study showing that local pressures in A_2 and A_4 in the hamster cheek pouch were about 50% and 30% of the systemic pressure (Davis *et al.*, 1981). Based on these studies, following estimation can be speculated: when the perfusion pressure gradient (ΔP) is 25 mmHg, local pressure gradient approximated 50% of ΔP in A_2 (12.5 mmHg) and 30% of ΔP in A_4 (7.5 mmHg). According to this estimation it is expected that large, 2nd order, arterioles may actually experience higher pressure gradient compared to small, 4th order arterioles.

Differential features of autoregulatory mechanisms in series-arranged arterioles in the hamster cheek pouch

One of the major findings of this study is that when perfusion flow and/or pressure increases in upstream feeding arteries, flow-induced dilation is the dominant regulatory mechanism in large arterioles (A_2); whereas, myogenic mechanisms play a major role in downstream, small arterioles (A_4). The prominent flow-induced dilation in A_2 seen in this study could be the consequence of higher shear stress gradient, when Q_p/P_p was elevated stepwise. However, data summarized in Table 12 indicate that this is not the case since the ratios of $\Delta T_{A_2}/\Delta T_{A_4}$ were less than one in all conditions. Likewise,

the local pressure gradient in smaller arterioles (A_4) should be lower (20%) than those larger arterioles (A_2), as estimated above. Therefore, the predominance of the myogenic responses seen in 4th order arterioles should not be the effect of larger driving forces, i.e. the pressure gradient. Hence, we postulated that the following factors may be responsible for this phenomenon: 1) large, 2nd order, arterioles have lower myogenic responsiveness compared to small, 4th order, arterioles; and 2) these large arterioles have higher sensitivity to mechanical stimuli, such as shear stress alterations.

That smaller arterioles are regulated by myogenic mechanisms was supported by an *in vivo* study of Meininger *et al.* (1987) in which a "box pressure" technique was used. In this technique, animals (rats) were "sealed" in a pressurized box while the exposed cremaster muscle was viewed on a pedestal outside the box in the air. Hence, the transmural pressure in the vasculature of the cremaster could be "controlled" and/or varied since the pressure inside the vessels of the cremaster were altered with the box pressure. They found that increases in box pressure had no effect on vessel diameter in 1st and 2nd order arterioles but caused vasoconstriction in 3rd and 4th order arterioles although blood flow in all of these arterioles was reduced when pressure in the box was increased. Therefore, under similar conditions as varied by the transmural pressure gradient, large (2nd order) arterioles exhibited a smaller myogenic responsiveness and/or myogenic gain compared to small (4th order) arterioles.

The concept that large arterioles were not as myogenic active as small arterioles also is in agreement with the report of Davis (1991) showing that in isolated cheek pouch arterioles, relative myogenic responsiveness increased with decreasing vessel size.

Although this conclusion was based on data from isolated arterioles and the behavior of

arterioles may be different when isolated from their vascular bed, it seems likely that in both isolated and *in situ* studies that small arterioles exhibited higher myogenic response, i.e. gains, as compared to large arterioles (Johnson, 1989; Kuo *et al.*, 1992; Davis, 1991).

In fact, the difference of myogenic responsiveness between A_2 and A_4 can be verified quantitatively. According to Jackson and Duling (1989) and Halpern *et al.* (1984), myogenic responsiveness can be quantified using a derivation, termed "myogenic index". It was defined as follows:

$$\text{Myogenic Index (MI)} = 100 \times (D_E/D_C) / \Delta P, \text{ which in turn} = \%D_C / \Delta P.$$

The final term on the right side of the equation is actually the slope of the normalized diameter-pressure ($\%D_C$ - P_p) relations. Notice that in our present study, when EDRF/NO release was inhibited with L-NAME perfusion, the $\%D_C$ - P_p relations shown in both 2nd and 4th order arterioles were presumably "pure myogenic". Under this condition we found that the slope of $\%D_C$ - P_p relation, thus the myogenic index, in 4th order arterioles had a significant higher value compared to 2nd order arterioles (-0.32 vs -0.10, negative signs indicating myogenic constriction). Moreover, in considering that the pressure gradient (ΔP) in A_4 was actually smaller than that in A_2 , as discussed earlier, the difference between the myogenic indexes in small versus large arterioles would be even greater. Hence the higher myogenic responsiveness and/or myogenic index in small, 4th order arterioles seems to be the major determinant for the prominent myogenic responses seen in these small arterioles.

Interactions between flow-induced and myogenic-induced mechanisms in series-arranged arterioles

One of our main conclusions is that autoregulation occurred in both large and small arterioles. This was based on the fact that the slopes of $\%D_C$ - P_p relationship obtained in control conditions were significantly different from those seen after dilating these same arterioles with SNP. As we know, in the condition of maximal dilation, i.e. vascular tone uncoupled and/or released, vessels should behave like elastic tubes that distend passively in response to an increased intramural pressure. In our previous study (Chapter 4), the dose-response curves showed that 10^{-6} M SNP produced maximal dilation in large (A_2) arterioles and 80% of maximal dilation in small (A_4) arterioles. These data supported the assumption that vascular tone in A_2 and A_4 can be sufficiently uncoupled by the suffusion of 10^{-6} M SNP. Indeed, the correlation between $\%D_C$ and P_p during SNP suffusion matched the classic characteristics of "passive distension", i.e. highly linearized $\%D_C$ - P_p relationship ($r=0.99$) with a positive slope, in both 2nd and 4th order arterioles. Hence, it is the significant differences between control and "passive" $\%D_C$ - P_p slopes that substantiated and confirmed the autoregulation in these large and small arterioles.

When comparing the $\%D_C$ - P_p relationships for different experimental conditions, interactions between flow-induced dilation and myogenic responses were evident in these series-arranged arterioles. As shown in Figures 21, 22, and 23, increases in perfusion flow were associated directly with a concomitant increase in perfusion pressure. Obviously the subsequent responses should reflect the influences from both stimuli, i.e. elevations in flow and/or pressure. Theoretically, the myogenic response to increases in

pressure should produce vasoconstriction; whereas, the flow-induced response has the potential to elicit vasodilation if the endothelium was stimulated to release dilating factors. Accordingly, when both pressure and flow increase, these two mechanisms are placed in opposition and the result would be the integration of both components. Indeed, this is exactly what we saw in this study. As illustrated in Fig. 24, a flow-dependent dilation occurred in large arterioles (A_2) under the control condition, as indicated by a positive but smaller than the "passive" $\%D_C-P_p$ slope. It is unlikely that these dilations are due to passive distensions since constrictions were observed after inhibiting the synthesis of EDRF/NO. The conversion of $\%D_C-P_p$ relationship from a positive correlation to a negative correlation after L-NAME perfusion signified a mediating role of EDRF/NO in this flow-induced dilation. On the other hand, the $\%D_C-P_p$ slope was significantly smaller than the passive $\%D_C-P_p$ slope obtained after SNP suffusion; this indicated an myogenic constrictor component under control conditions. Hence, it is the integration of both flow-induced and myogenic- components that give rise to the control $\%D_C-P_p$ responses, thus the autoregulation. In small arterioles (A_4), the flow-induced effect was depressed strongly by the myogenic component at control condition. Therefore, an overall constriction was observed. The dilating component could be distinguished only at a higher level of perfusion flow (Q_p-3) when these arterioles dilated towards, but did not reach, their basal level. Likewise, this dilation was abolished completely after L-NAME perfusion implying the involvement of EDRF/NO in this dilating component. However, since the myogenic mechanism was the major component under the control condition, the inhibition of EDRF/NO release did not significantly alter the slope of $\%D_C-P_p$ relation. This suggests that the myogenic response in these small

arterioles is independent of the EDRF/NO pathway.

The interaction between pressure-induced myogenic responses and flow-induced dilator responses has been studied *in vitro* using arterioles isolated from porcine coronary bed. Kuo *et al.* (1991) reported that flow-mediated dilation competed with myogenic constriction when flow and pressure were elevated; whereas, flow potentiated myogenic dilation when intramural pressure was decreased. Their technique allowed them to change flow without altering intramural pressure or to change intramural or transmural pressure while stabilizing flow, thus separating these two stimuli. Under these conditions, the myogenic responses, vasoconstriction, seemed to be dominant when pressure was altered; whereas, a flow-induced responses, i.e. dilation, were the most important component at a normal *in vivo* level of intramural pressure. Although effects of pressure and flow were not dissociated technically in our system, evidence from their study coupled with results seen in this study strongly supported the hypothesis that flow competed with pressure when both flow and pressure were elevated.

According to Bevan and Joyce (1990), flow-mediated responses do not always produce dilation and the nature of flow-induced responses is dependent on the pre-tone, the level of the vascular tone prior to the onset of the flow gradient. Using a resistance artery preparation isolated from the rabbit ear they found that at a certain level of vascular tone, increase in flow produced neither constriction nor relaxation. They termed this tone level as "null point". Moreover, their results have shown that no matter what the pre-tone was in a particular artery, the vessel tended to respond to changes in flow by shifting its vascular tone level in the direction of the null point. This means that if the pre-tone were lower than the null point, a flow-induced constriction would occur, thus

raising vascular tone towards the null point. Our results seem to be incongruent with their findings. In large (A_2) arterioles with low pre-tone as indicated by a smaller response to vasodilators, the increase in perfusion flow produced a dilation where one might have expected a constriction according to Bevan and Joyce. Furthermore, the small (A_4) arterioles with higher pre-tone constricted in response to increased flow which also contradicts the "null point" hypothesis. There are at least three plausible interpretations for these discrepancies. First, there may be differences in methodologies, e.g. isolated vessel versus intact vascular bed, pharmacologically pre-constricted versus intrinsic vascular tone, etc. Secondly, for the "null point" hypothesis to be applicable in our preparation, the null points for large and small arterioles would need to be disparate. In other words, the null point for large arterioles would need to be even lower than the already low pre-tone in order to have the dilation to occur. And likewise, for the small arterioles with already high pre-tone, the null point would need to be even higher for a constriction were to bring vascular tone up towards the null point level. Lastly, the responses seen in our study were not purely flow-induced, but a resulting integration of flow- and pressure-induced responses in which myogenic component could mask or counteract the effects of flow. Regardless of these discrepancies, the regulatory mechanisms in upstream arterioles (A_2) and downstream arterioles (A_4) were distinctive and they were determined by the integration of multiple factors including the function of the endothelium, the gain of myogenic responses, the pre-tone of the particular arteriole and the sensitivity of mural cells to mechanical stimuli such as shear stress.

Involvement of EDRF/NO pathway in flow-induced response

Phol and Lamontagne (1991) reported that inhibition of EDRF synthesis by

N^o-nitro-L-arginine resulted in abolition of the flow-dependent dilation of coronary resistance vessels using an isolated, perfused rabbit heart. Meanwhile, Kuo *et al.* (1991) also found that flow-induced dilation was abolished by N^o-monomethyl-L-arginine or by mechanical removal of endothelium using coronary arterioles isolated from pigs. In an *in vivo* preparation of rat cremaster, however, Koller and Kaley (1990b) demonstrated that flow-induced dilation was mediated by prostaglandins rather than EDRF/NO.

Although the mediator released upon increasing blood flow may be different from tissue to tissue, these studies all demonstrated that the flow-induced response is dependent upon intact, functional endothelial cells. Contradictory data, however, also have been reported. Bevan and Joyce (1990) and Garcia-Roldan and Bevan (1990) demonstrated that in isolated arteries from rabbit ear and arteries isolated from pial circulation, the flow-induced responses were independent of the endothelium. Our present experiments were conducted in arterioles from an intact vascular system in which the endothelial function has been preserved well since "normal" Ach responses were evident. Therefore, the abolished flow-induced dilation after intra-arterial administration of L-NAME, an L-Arginine/EDRF/NO pathway blocker signified that the endothelium was involved in this flow-induced dilator response; while, EDRF/NO was most likely the mediator responsible for the subsequent dilation.

Randall *et al.* (1991) reported that in a mesenteric vascular beds of Wistar-Kyoto rat, the destruction of the endothelium significantly increased the slope of the pressure-flow relation implying a significant role of endothelium in the autoregulation of the blood flow in this particular vascular bed. Likewise, in a study using an isolated buffer-perfused rabbit ear preparation, it was found that the pressure-flow relation was

sigmoidal in shape (indicating an autoregulation) with controlled-pressure but not controlled-flow perfusion (Griffith and Edwards, 1990). It was therefore concluded that myogenic autoregulation of flow may be inversely related to EDRF/NO activity. Consistent with these findings, the present study illustrated that the inhibition of EDRF/NO synthesis significantly altered flow-induced diameter changes as well as the $\%D_C-P_p$ slope in large (A_2) arterioles thus indicating a significant role of EDRF/NO mediated, flow-induced dilation in autoregulation for this segment of arterioles. In 4th order arterioles, however, the myogenic response was predominant at control and there were no significant differences found between $\%D_C-P_p$ slopes obtained under control and L-NAME perfused condition suggesting that in this particular segment of arterioles, the predominant myogenic autoregulation is independent of the EDRF/NO pathway.

Furthermore, it was observed in our study that intra-arterial applications of L-NAME did not alter the basal diameter of either arterioles (Tables 9, 10, diameters at Qp-1). This is in agree with the report of Kuo *et al.* (1991) showing that resting arteriolar tone was not significantly altered after mechanical removal of endothelium. As discussed earlier, vascular tone that exists prior to the onset of the flow/pressure gradient may affect subsequent autoregulatory responses. Hence, the fact that basal diameter of these arterioles, thus their basal tone, was not affected by L-NAME perfusion is particularly important since this proves that altered diameter-pressure relationships seen after L-NAME perfusion were not the consequence of variations in the level of basal vascular tone, i.e. the vascular smooth muscle may be operating still at the same point on the length (diameters)-tension curves.

Relevances to the physiological conditions

The different features of 2nd and 4th order arterioles in autoregulatory responses could have significant physiological implications. As Joyner and Davis (1987) and Joyner *et al.* (1981) reported, A_3 - A_4 arterioles are one of the two major intravascular pressure dissipation points in the circulation of the cheek pouch and might be the optimal point for the regulation of blood flow and/or pressure. Therefore, it may be crucial for these small arterioles to be more sensitive to myogenic stimuli for the purpose of having fine pressure regulation, maintaining a constant perfusion pressure for tissue exchange, and providing a constant flow to meet the tissue metabolic demand. The dominant flow-induced dilator mechanism in upstream 2nd order arterioles helps to reduce the shear stress on these vessels, thus protecting the endothelium from the damage during increases in metabolic demand, such as exercise. Meanwhile, it will lower the resistance of the flow to ensure the perfusion of the downstream tissues.

Data obtained in reversed procedure (Q_p -R in Tables 9, 10, 11) demonstrated that in control condition, vascular diameter of both 2nd and 4th order arterioles recovered to their basal diameter after Q_p was returned from higher level to the initial level. This suggests that both large and small arterioles are able to autoregulate blood flow regardless the direction of flow/pressure alterations in a ΔP range of 50 mmHg. This is particularly important for tissues to maintain constant flow and proper local hydraulic pressure during the hemorrhagic shock or upstream occlusion.

Conclusions

In summary, large (A_2) and small (A_4) arterioles in the hamster cheek pouch are autoregulated by mechanisms involving both an endothelial-dependent, flow-induced

dilation and a potential endothelial-independent myogenic constriction. Under the normal local flow condition, upstream 2nd order arterioles exhibited predominantly flow-induced dilator responses; whereas, downstream 4th order arterioles, displayed primarily myogenic vasoconstrictor responses to the elevations in perfusion flow/pressure.

EDRF/NO is most likely the mediator responsible for the flow-induced dilation; nevertheless, the myogenic responses seem to be independent of EDRF/NO pathway.

Moreover, these regulatory mechanisms are independent of blood borne hormones, cells or vasoactive agents.

CHAPTER 7

SUMMARY, CONCLUSIONS AND PERSPECTIVES

In summary, the role of the endothelium in modulating vascular tone and the distribution of the microvascular blood flow seems to be heterogeneous in the arteriolar segments of the series-arranged network in the hamster cheek pouch. This study is the first to demonstrate the heterogeneity of EDRF activity in upstream, 2nd order arterioles and downstream, 4th order arterioles. Also, it is the first study to illustrate the distinct regulatory mechanisms in large, 2nd order, and small, 4th order, arterioles during mechanical stimulations (e.g. alterations in flow/or pressure) in an intact microvascular bed.

The integrity of the endothelium in these small arterioles contributes to agonists-induced vasoactive responses evoked not only by the dilators (e.g. Ach) but also by constrictors, such as angiotensin(s). Therefore, the endothelium plays a leading role in balancing the responses of locally released, or blood born constrictors and dilators which in turn controls vascular resistance. It is the final integrated signal that altered the vascular diameters in a certain direction. This is particularly beneficial to the adjustments of resistance in these small arterioles since 3rd and 4th order arterioles are one of the two sites that produce large pressure drops in the vasculature of the hamster cheek pouch (Davis *et al.*, 1986; Joyner and Davis, 1987).

Flow-induced responses have been suggested to be involved in many procedures including: 1) changing resistance during metabolic hyperemia (Lamping and Dole, 1988), 2) augmenting collateral flow when a feed artery is occluded (Smiesko *et al.*,

1989), 3) promoting regional vascular growth during persistent hyperemia (Rodbard, 1975), 4) modulating neurogenic vasomotor tone (Tesfamariam and Cohen, 1988), and 5) coordinating the aggregate hydrodynamic properties of a vascular network (Griffith *et al.* 1987). This study suggests that interactions between flow- and pressure-induced responses occur *in vivo*. The sensitivity or the responsiveness in these series-arranged arterioles, however, is different when activated by mechanical stimuli. The flow-induced dilation appears to be dominant in large, 2nd order arterioles and EDRF is most likely the mediator in this response. In small, downstream, 4th order arterioles, on the other hand, the myogenic control has a much higher gain that eventually overcomes the metabolic effect or flow-induced responses. Furthermore, myogenic control in these small arterioles seems to be due to vascular smooth muscle and it is independent of the endothelium. These differential features in large and small arterioles may have significant implications. For instance, during exercise, peripheral blood flow increases dramatically and leading to a dilation of upstream arterioles which helps to assure an efficient blood supply to the downstream vessels to meet the tissue demand and to lower the shear stress on these arterioles. The downstream arterioles, however, tend to constrict due to myogenic control mechanisms; thus maintaining a certain hydrostatic pressure for the exchange in accord with the increased metabolic demands. Hence, the interaction of pressure- and flow-induced responses may play an important role in fine adjustments and moment-by-moment control of blood flow. It is conceivable that during pathological conditions, e.g., hypertension, diabetes, and atherosclerosis, the increased vascular tone and/or the abnormal endothelial function could attenuate the effect of flow on vascular resistance in the microcirculation and may result in inadequate oxygen supply during

intense metabolic demands.

This study has explored the differential roles of the endothelium in mediating vasoactive responses to hormonal/humoral or mechanical stimulation in series-arranged arterioles. Mechanisms that yield these differential responses, however, need additional studies. Further investigation can be conducted using the maneuver that physically removes endothelial cells from the vascular wall, such as air bubble technique used in the study of Buchanan *et al.* (1987). This will provide direct evidences demonstrating more specifically the role of endothelial cells involved in different responses; therefore, ruling out the interferences from the other mural cells, such as pericytes and mast cells, which have also be reported to have important influences in the local environments (Miller *et al.* 1992).

Other methods, such as the identification of nitric oxide synthase (NOS), also could be considered to understand further the differences seen in the transduction pathways for EDRF activity in different segments of series-arranged arterioles. As introduced in the first chapter, the synthesis of NO from arginine in the endothelium is catalyzed by an NADPH and calcium dependent enzyme, Nitric Oxide Synthase. The distribution of this enzyme could be an indication of the chemical resources necessary for the L-arginine ~ EDRF pathway, and therefore, could answer part of the mysteries. It is reported by several groups that NOS and neuronal NADPH diaphorase are identical in brain and peripheral tissues (Dawson *et al.*, 1991; Hope *et al.*, 1991). Therefore, NOS could be located by NADPH diaphorase histochemical staining in whole mount tissues using nitroblue tetrazolium, a NADPH diaphorase specific dye.

As reviewed in the first chapter, the intracellular events that link receptor

stimulation to the release of EDRF are related closely to the elevation of intracellular calcium. In other words, a rise of intracellular free calcium is probably a prerequisite for the release of EDRF (Lückhoff and Busse, 1986). In addition, the enzyme implicated in the formation of EDRF/NO from endothelial cells is reported to require NADPH and divalent cation(s) and Ca^{2+} is most likely involved (Palmer and Moncada, 1989).

Therefore, alterations in intracellular calcium could be one of the keys that opens the door to the cellular transduction pathway(s) residing behind the different mechanisms governing the local blood flow regulation in series-arranged arterioles. Hence, the next step of this research could be the investigation of intracellular calcium changes in endothelial dependent responses evoked by agonist- or flow/ pressure- stimulations in different segments of the arterioles in the intact microvascular bed. This can be accomplished by using Fluo-3, a newly developed fluorescent calcium indicator, in combination with pump-perfusion of the *ex vivo* cheek pouch preparation. Fluo-3 allows parallel read-out or imaging of spatially heterogeneous signals and it can be loaded into the cytoplasm of a large variety of intact cells without any puncturing or even temporary disruption of the plasma membrane (Tsien, 1988; Kao *et al.*, 1989); thus, it makes the investigation feasible. Hopefully, data demonstrating the transcellular movement of intracellular calcium would help to interpret the phenomena of the heterogeneous distribution of EDRF/NO in these different segments of the series-arranged arterioles and clarify the role of the endothelium.

In conclusion, the local control of blood flow and autoregulatory behavior in the microcirculation is distinctive at different levels of the vasculature; whereas, the differential role of the endothelium in discrete segments of series-arranged arterioles

seems to be the determinant for these differences. In small arterioles (A_4), the endothelium is required for acetylcholine-induced dilation, and this acetylcholine-induced dilation involved the release of EDRF/NO from the endothelium. In addition, the integrity of the endothelium is required for the local angiotensin conversion in these small arterioles as well. In large arterioles (A_2), however, Ach-induced dilation and local angiotensin conversion appears to be independent of the endothelium. When sensing the mechanical stimuli (such as shear stress or stretch), the endothelium-mediated, flow-induced dilation is dominant in large arterioles (A_2). In small arterioles (A_4), however, the myogenic mechanism for local control (which appears to be endothelial-independent) plays predominant role in response to mechanical stimuli, and the myogenic mechanism has the power to override the flow-induced, EDRF-mediated dilation. Therefore, the function of the endothelium does not appear homogenous throughout the arteriolar portion of these microcirculations.

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